THE ROLE OF THE KAPPA OPIOID RECEPTOR SYSTEM IN FOREBRAIN DEPENDENT ASSOCIATIVE LEARNING

BY

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DISSERTATION

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Abstract

The opioid receptor system has been shown, through various lines of evidence, to be involved in pain processing, addiction, and learning and memory. It has been previously established that the mu opioid receptor (MOR) is intimately involved in the acquisition and consolidation of memories. While these studies have provided valuable insight into the role of MOR in learning, researchers have recently begun to elucidate a role for the kappa opioid receptor (KOR) with learning. Previous reports have demonstrated that KOR and its ligands are capable of modifying complex learned behaviors in paradigms such as water maze and fear conditioning. While these studies have established an important foundation suggesting that KOR plays a role in learning and memory, clear evidence for, and an understanding of the mechanistic role of KOR in learning is still lacking. To explore the role of KOR in learning, we have used the associative learning paradigm whisker trace eyeblink (WTEB) conditioning.

With WTEB conditioning animals are trained to associate a neutral conditioned stimulus (CS - whisker stimulation), following a stimulus free trace interval with a salient unconditioned stimulus (US – periorbital shock). With successive CS-US paired presentations, the subject begins to elicit a conditioned response (CR - eyeblink) to the CS, prior to US onset. Acquisition for this paradigm has been demonstrated to be forebrain dependent as removal of either the hippocampus or the neocortex can either hinder, or entirely block acquisition of the association (Solomon et al., 1986, Moyer et al., 1990, Kim et al., 1995, Weiss et al., 1999, Takehara et al., 2002, Galvez et al., 2007).

Using this paradigm, the following thesis outlines a series of experiments designed to examine opioid and specifically KOR involvement in forebrain dependent learning mechanisms. To do this we first demonstrated in Chapter 2 that a global opioid antagonist, naloxone,

administered intraperitoneally effectively blocks acquisition of the WTEB association. Further examining this relationship, in Chapter 3, systemic and local somatosensory neocortical injections of the KOR specific antagonist, NorBNI, demonstrate that blocking KOR results in a similar impaired acquisition. Following this pathway downstream, Chapter 4 outlines a phasic property of KOR in WTEB conditioning, in that early-phase antagonism causes deficits in acquisition of the association, while late-phase antagonism causes deficits in memory consolidation. Lastly, Chapter 5 demonstrated a significant increase in the amount of the dynorphin precursor peptide prodynorphin (PD) in the somatosensory cortex during and immediately following WTEB training. Collectively, the findings presented in this dissertation outline a novel role for KOR in the acquisition and consolidation of forebrain dependent associative memories while, providing additional insight into the underlying neocortical mechanism mediating our ability to learn and consolidate information.

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Chapter 1 - Introduction

The opioid system is extensively connected throughout the central and peripheral nervous systems (Stein, 2003) and has a long history of involvement in various neurobiological functions such as pain (Ballantyne, 2006), learning and memory (Miguez et al., 2014), and reward (Berridge and Kringelbach, 2015). It comprises four opioid receptor subtypes (μ, κ, δ, and opioid receptor like (ORL)), that are activated by one of four endogenous ligands (endorphins (μ), dynorphins (κ), enkephalins (δ), and nociceptins (ORL)) (Stein et al., 2003). These endogenous ligands have been shown to bind preferentially to one receptor (see parentheses after each ligand above), but are similar enough to have overlapping binding profiles. For an extensive review of these receptors and their endogenous ligands see (Feng et al., 2012). Historically, the μ opioid receptor has been the most extensively investigated and although its involvement in various processes will be discussed when relevant, this dissertation will primarily focus on the κ opioid receptor (KOR). In exploring the role of the opioid system in the brain, its involvement in the reward pathway has been one of the most extensively investigated.

The Reward Pathway

The reward pathway, a prominent system for motivating behavior, consists of various brain regions and neurotransmitters, but is classically defined by dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and the prefrontal cortex (PFC). This portion of the reward pathway has been shown to be important in that mesotelencephalic dopamine (DA) pathway lesions hinder the acquisition of reward-based learning tasks, such as intracranial self-stimulation (Fibiger et al., 1987). Similarly, in operant conditioning, DA signaling initially increases during the presentation of a reward following a cue; however, with substantial cue-reward pairings, there is a temporal shifts in dopamine DA

concentrations towards the onset of the cue stimulus (Day et al., 2007). Chang et al. has also demonstrated the DA in the VTA to be responsible for mediating both positive and negative reward prediction errors (Steinberg et al., 2013, Chang et al., 2016). What both of these studies indicate is that DA signaling within the VTA is capable of mediating the formation of new reward-mediated associative memories. Collectively, these studies establish an important role for DA and the reward system in the acquisition of various reward-motivated tasks.

Interestingly, DA regulation in this part of the reward pathway is modulated through opioid receptor stimulation. GABAergic cells within the VTA provide a tonic inhibition on DA transmission; however, μ-opioid receptors (MOR) on the axon terminals of those cells, when activated, remove that inhibition, therefore increasing DA transmission to the NAc (Johnson and North, 1992). Similarly, MORs have been shown to be localized to medium spiny GABAergic neurons in the NAc, suggesting their role in directly modulating inhibitory activity within the region (Svingos et al., 1997). A simplified summary of this localization is outlined in Figure 1. Interestingly, it has been established that KORs located on cell bodies of VTA DA neurons and axon terminals of the same neurons in the NAc serve to decrease the amount of DA release in the NAc (Spanagel et al., 1992). This KOR localization in the VTA is further supported by the fact that suppression of KOR expression on VTA DA neurons inhibits KOR-induced conditioned place aversion (CPA) (Ehrich et al., 2015). The aforementioned receptor profile allows for a balancing of the reward pathway such that MOR agonists increase while KOR agonists decrease dopamine transmission. This results in MOR activation generally having reinforcing/rewarding effects on many behaviors that are blocked or in some cases completely reversed with KOR activation. Behaviorally, rodents will readily self-administer MOR agonists directly into the NAc (Goeders et al., 1984, Self and Stein, 1993); while administration of KOR agonists into the VTA

or mPFC are capable of inducing CPA (Tejeda et al., 2013, Ehrich et al., 2015). Interestingly, blocking KOR transmission systemically or directly in the VTA also reduces CPA (Ehrich et al., 2015, Kelsey et al., 2015). These studies collectively suggest that KOR activation in the reward pathway alters the amount of DA release resulting in a negative effect on reinforcing/rewarding behaviors.

In exploring the role of KOR in learning, many studies have again focused on its role in DA transmission throughout the reward pathway (Schlosburg et al., 2013, Kelsey et al., 2015). This can be noted especially in studies that have utilized reward- and withdrawal-induced learning environments (Kelsey et al., 2015), conditioned place aversion (Tejeda et al., 2013), and conditioned place preference (Bolanos et al., 1996). Bolanos and colleagues demonstrated that a pre-injection of a KOR agonist reduces conditioned place preference in rodents exposed to morphine (Bolanos et al., 1996). Similarly, it has been shown that antagonists given during a withdrawal period of morphine dependence are capable of reducing conditioned place aversion (Kelsey et al., 2015). While these papers did not locally inject the drugs, it was hypothesized that the KOR effect was being mediated through the aforementioned reward pathway. Similarly, rats trained to self-administer heroin (MOR agonist) displayed reduced escalation, decreased motivation to consume, and reduced withdrawal-induced anxiety-like symptoms when dosed systemically or locally into the NAc with NorBNI, a KOR specific antagonist (Schlosburg et al., 2013). Likewise, KOR specific antagonism has been shown to have similar effects when co-administered with other drugs that increase DA release in the reward pathway. For instance, rats trained to self-administer alcohol show similar attenuations of the behavior with intra-NAc injections of NorBNI (Nealey et al., 2011). Cocaine conditioned place preference and cocaine-induced DA transmission are also attenuated with intraperitoneal injection of a KOR agonist

when given 15, but not 60 minutes before cocaine administration (Ehrich et al., 2015). These studies have argued that these behavioral effects are due to modulation of KOR in the NAc altering DA transmission. While these KOR modulating drugs are hypothesized to be working through this mechanism, what these papers fail to note is how the high concentrations of KOR in other areas of the brain may be altering learning mechanisms and the observed behaviors.

Learning

Like its role in the reward pathway, there are many studies exploring the role of the opioid system in modulating non-reward mediated learning. It is important to note that when exploring the role of the opioid system in non-reward mediated learning pathways, it is conceptually impossible to design a learning paradigm that cannot be influenced by the reward pathway. Thus in exploring this question, learning paradigms that are not typically used and are presumed to not be primarily driven by reward-based learning are explored. For example, when MOR is knocked out in mice, they display a greater number of errors in radial arm maze and increased escape latency in water maze (Jamot et al., 2003, Jang et al., 2003). Likewise, injections of the MOR antagonist beta-funaltrexamine into the hippocampus causes deficits in the acquisition of a water maze task, as measured by escape latency and target-quadrant time for probe trials across 5 days (Meilandt et al., 2004). Additionally, deletion of MOR in mice causes an increase in the survivability and number of new cells in the hippocampal dentate gyrus, a processes believed to be critical for, and facilitate learning and memory consolidation (Jessberger et al., 2009, Anderson et al., 2011, Cominski et al., 2014). While these studies demonstrated MOR to play a role in learning, less is known regarding the involvement of KOR.

Autoradiography studies have demonstrated KOR to be ubiquitously localized throughout the brain, with a high concentration of dynorphin (DYN)-containing cell bodies throughout the

limbic system and regions important for learning and memory such as the neocortex, hippocampus, amygdala, and thalamus (Jomary et al., 1988, Slowe et al., 1999). Consistent with this localization, KOR activation via either age- or injection-induced increases in DYN expression in the hippocampus causes impaired acquisition for the water maze task as measured by escape latency (Jiang et al., 1989, Sandin et al., 1998). In contrast, KOR activation via intracerebroventricular DYN injections have also been demonstrated to have protective effects against ischemic-induced Y-maze deficits (Itoh et al., 1993c). Likewise, in a task in which a rodent is trained to seek alcohol and subsequently has that behavior extinguished, local KOR agonist injections into the thalamus demonstrate an inability to reform the association after extinction relative to saline controls (Marchant et al., 2010). These studies suggest that KOR activation is capable of modifying various learned behaviors; however, studies have shown that, blocking KOR activation can also have an effect on learning. For instance, intracerebroventricular injections of the KOR antagonist NorBNI can impair acquisition for contextual fear conditioning (Fanselow et al., 1991). In interpreting these findings, Fanselow and colleagues primarily argued for the role of KOR in pain processing. Additionally, withdrawal-induced conditioned place aversion is blocked with systemically administered KOR antagonists (Kelsey et al., 2015), demonstrating that KOR cannot be thought of as a simple one-way regulator of learning. Although the specific role for KOR in learning is not well understood, these studies collectively suggest that KOR, like MOR plays a modulatory role in specific types of learning and memory.

Other studies seeking to understand the role of KOR in learned behaviors have utilized knockout models. Bilkei-Gorzo and colleagues have demonstrated that prodynorphin (pDYN) knockout mice demonstrate enhanced freezing rates relative to intact controls (Bilkei-Gorzo et

al., 2012). Similarly, pDYN knockout mice have been shown to display reduced age-induced deficits as measured by acquisition and retention in a water maze task (Nguyen et al., 2005). At the receptor level, it has been demonstrated that mice lacking KOR show enhanced performance in both the Morris water maze as measured by escape latency, as well as the Radial arm maze as measured by working memory errors (Jamot et al., 2003). While these studies provide interesting insights into the role of the opioid system in learning and memory, they have the important caveat of utilizing knockout models. This poses a problem as organisms can develop compensatory mechanisms as they age in the absence of specific proteins hindering detection of the actual role of these receptors in learning.

While much work has been done establishing the role of KOR in modulating stimuli salience in significantly aversive learning paradigms (via fear conditioning, water maze, or food deprivation in radial arm maze) or reward pathway-mediated learning (morphine-, heroin-, cocaine-, alcohol-induced reward/withdrawal), few studies have explored the mechanistic role of KOR directly affecting learning mechanisms. While we recognize that any animal learning paradigm will induce some degrees of stress, it has been suggested that eyeblink conditioning is only marginally stressful, in that serum corticosterone levels are increased in all groups exposed to the novel training environment, but not significantly higher in groups that underwent training (Shors et al., 1992). To our knowledge, one group that has attempted to study the mechanistic role of KOR on learning demonstrated that a systemically administered KOR agonist attenuated acquisition for delay-eyeblink conditioning in rabbits. This effect on acquisition was blocked with the systemic opioid antagonist, naloxone, administered at the same time as the drug (Schindler et al., 1986, Schindler et al., 1987). This effect on eyeblink conditioning was further examined by our laboratory in a follow-up study looking at the effect of NorBNI on the

acquisition of trace eyeblink conditioning in mice. As further discussed in Chapter 3, our study demonstrated that both systemic and intra-S1 injections of the KOR antagonist NorBNI caused an attenuation of the acquisition for whisker-trace eyeblink conditioning (WTEB) (Loh and Galvez, 2015).

Whisker Trace Eyeblink

WTEB is an associative learning paradigm that utilizes the association of a neutral conditioned stimulus (CS; whisker stimulation) with a salient unconditioned stimulus (US; eyeshock) following a stimulus free trace interval. This paradigm has been utilized in multiple species and has been shown to be dependent upon KOR containing forebrain structures such as the neocortex, hippocampus, and thalamus (Solomon et al., 1986, Moyer et al., 1990, Kim et al., 1995, Weiss et al., 1999, Takehara et al., 2002, Galvez et al., 2007). Interestingly, one study investigating the glucose recruitment in brain regions during eyeblink conditioning with 2-deoxyglucose demonstrated no significant increases in two classic reward pathway brain regions (VTA, NAc), suggesting that these brain regions do not exhibit increased activation with learning in this task (Plakke et al., 2009). Thus further suggesting that WTEB is a suitable paradigm for examining the role of KOR in non-reward pathway brain regions. Most recently, this paradigm has been established to induce plasticity in the somatosensory cortex (S1), a likely site mediating consolidation of the WTEB association and a brain region not part of the reward pathway (Chau et al., 2013, Chau et al., 2014b). Note, these studies do not suggest that KOR in the reward pathway cannot alter WTEB learning; however, this paradigm and specifically KOR mediated activation within S1 offers a tool for examining the role of KOR in non-reward mediated learning mechanisms.

Ligand-Dependent Signaling of KOR

In exploring the potential role of KOR in learning it is first critical that we fully understand its known activation cascade and biochemical properties. KOR is a g-protein coupled receptor (GPCR) that is activated through its endogenous ligand DYN (Figure 2). The expression profile of DYN shows a near ubiquitous localization within regions such as the neocortex, hippocampus, NAc, hypothalamus, ventral striatum, amygdala and spinal cord (Khachaturian et al., 1982, Fallon and Leslie, 1986). Importantly for our research, preprodynorphin (PPD) has been found expressed within GABAergic interneurons in the somatosensory neocortex (Sohn et al., 2014). Like all opioid receptors, KOR activation can be broken down into a two stage process; an initial ligand-dependent kinase activation stage, and a subsequent, GRK3/arrestin-dependent transcription factor stage that results in receptor internalization and downregulation. In stage-1 DYN binds to KOR activating $G\alpha_i$ which then inhibits the production of cAMP via inhibition of adenylyl cyclase (Taussig et al., 1993) and activates various downstream mitogen activated kinases such as MAPK, early ERK1/2, PI3K, PKC and P38 (Belcheva et al., 1998, Xu et al., 2007). These kinases and their role in learning will be discussed further below. In addition to activating $G\alpha_i$, KOR stimulation activates $G\beta\gamma$. Upon activation, $G\beta\gamma$ dissociates and alters the conductance of calcium channels and inwardly rectifying potassium channels on the membrane (Spencer et al., 1997, Benians et al., 2003). This process allows for tight regulation of transmembrane ion flux. Upon chronic activation, in stage-2 KOR becomes phosphorylated due to the phosphorylation of GRK-3 (Appleyard et al., 1999), which then allows for the receptor to phosphorylate arrestin (Lohse et al., 1992). Upon activation, arrestin goes on to phosphorylate/activate the downstream transcription factors pCREB via ERK 1/2 (Bruchas et al., 2008) and zif268 via p38 (Bruchas et al., 2006). Chronic

ligand mediated KOR activation also causes arrestin-dependent receptor internalization through the GTPase, dynamin, and its ability to activate clathrin-mediated internalization (Zhang et al., 1996) and reduction in KOR activity (Bhargava et al., 1989). This biphasic activation pattern emphasizes the complexity of the receptor and may provide insight into the inconsistent pattern of activity seen in learning. To further break down the processes that KOR goes through in its ligand-dependent activation, it is imperative to focus on each stage.

In stage-1 KOR stimulation, as discussed above, results in the activation of various kinases (MAPK, early ERK1/2, PI3K, PKC and P38) that have been shown to be involved in the acquisition of various learning tasks. For instance, following associative learning, MAPK is upregulated in the hippocampus and cerebellum, and when blocked inhibits acquisition for fear and eyeblink conditioning (Atkins et al., 1998, Zhen et al., 2001). Similarly, a reduction in hippocampal ERK1/2 activity impairs contextual fear conditioning in mice (Shalin et al., 2004). PI3K activation in the CA3 region of the hippocampus is required for acquisition of conditioned place preference (Cui et al., 2010) and PKC inhibition in the hippocampus disrupts memory formation for Morris water maze as measured by escape latency over 16 consecutive trials (Bonini et al., 2007). Likewise, ERK1/2 is elevated in the hippocampus, while p38 is elevated in both the hippocampus and the cerebellum for up to 180 minutes following delay eyeblink conditioning (Zhen et al., 2001). These studies clearly demonstrate that these kinases modulate learning; thus further suggesting that KOR mediated activation of these kinases alters learning; a hypothesis further explored in Chapter 4.

Stage-2 of KOR stimulation results in the activation of the transcription factors pCREB and Zif268 that have also been shown to be involved in learning, particularly in memory consolidation. Genetic or pharmacological inhibition of CREB decreases retention of water

maze learning, while showing no effect on acquisition (Guzowski and McGaugh, 1997, Kida et al., 2002). Likewise, zif268 expression is increased in the medial prefrontal cortex (mPFC) following water maze training, with the most robust increase occurring 30 days after initial training (Barry et al., 2016). In addition, zif268 knockout mice show impaired water maze consolidation, and conditioned taste aversion. Interestingly, this effect was not seen upon initial assessment, but rather observed after 48 hours for water maze training and 24 hours for conditioned taste aversion (Jones et al., 2001). Interestingly, zif268 knockout mice also displayed impaired late long term potentiation and consolidation of novel object recognition (Jones et al., 2001). These studies demonstrate that the transcription factors CREB and zif268 do not appear to be critically involved in initial acquisition, but rather help mediate long term memory consolidation; thus further suggesting that KOR mediated activation of these transcription factors would alter long term memory consolidation; further explored in Chapter 4.

Proposal

The studies mentioned above suggest that KOR plays a role in learning and memory; however, clear evidence for, and an understanding of the mechanistic role of KOR in learning is still lacking. While previous studies have begun to elucidate the mechanistic role of KOR in learning, they are often more interested in its modulating effect within other systems such as stress and reward. Although these studies are important, it is equally imperative to understand the role that KOR modulation could be having directly on learning mechanisms. In the following chapters, we will provide evidence suggesting that there is a general role for opioids in the acquisition of WTEB training (Chapter 2). Furthermore, our subsequent studies will strongly suggest that while there is a general opioid effect, KOR in S1 specifically plays a role in modulating acquisition for the WTEB association (Chapter 3). Then in chapter 4 we will

investigate the specific role for each of the two stages of KOR activation with respect to WTEB conditioning. Finally, Chapter 5 will outline the upstream mechanism(s) of KOR activation during WTEB. I believe this thesis examines both up and downstream roles for the KOR system as they pertain to WTEB, and will provide a deeper understanding of neocortical mechanisms of associative learning.

Figures

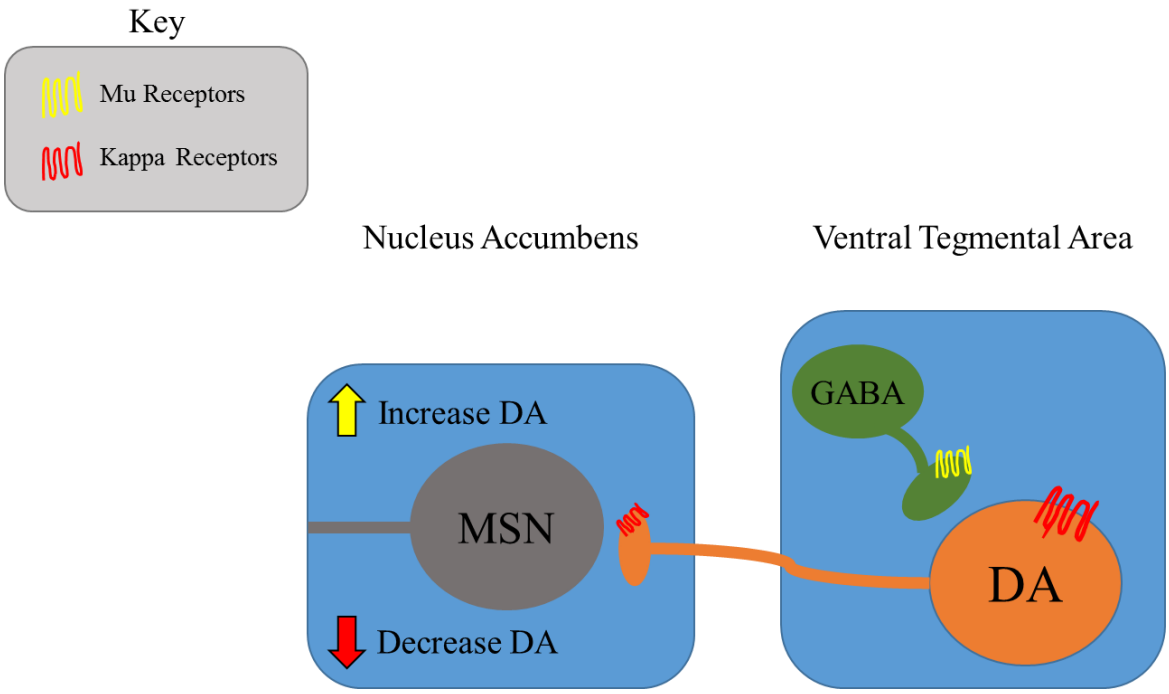

Figure 1: Schematic representing relevant locations of Kappa (KOR) and Mu opioid receptors (MOR) in mesolimbic pathway. While MORs are located on the axon terminals of GABAergic interneurons, KORs are located on the cell bodies and axon terminals of dopaminergic (DA) cells. The DA cells synapse onto medium spiny neurons (MSN) in the nucleus accumbens. Upstream of KOR in the ventral tegmental area are dynorphin projections from various locations in the limbic system. Arrows in the Nucleus Accumbens indicate effect of stimulation of MOR (yellow) or KOR (red) on DA transmission. Additionally, not pictured are MSN dynorphin projections that serve as negative regulators of ventral tegmental DA activity.

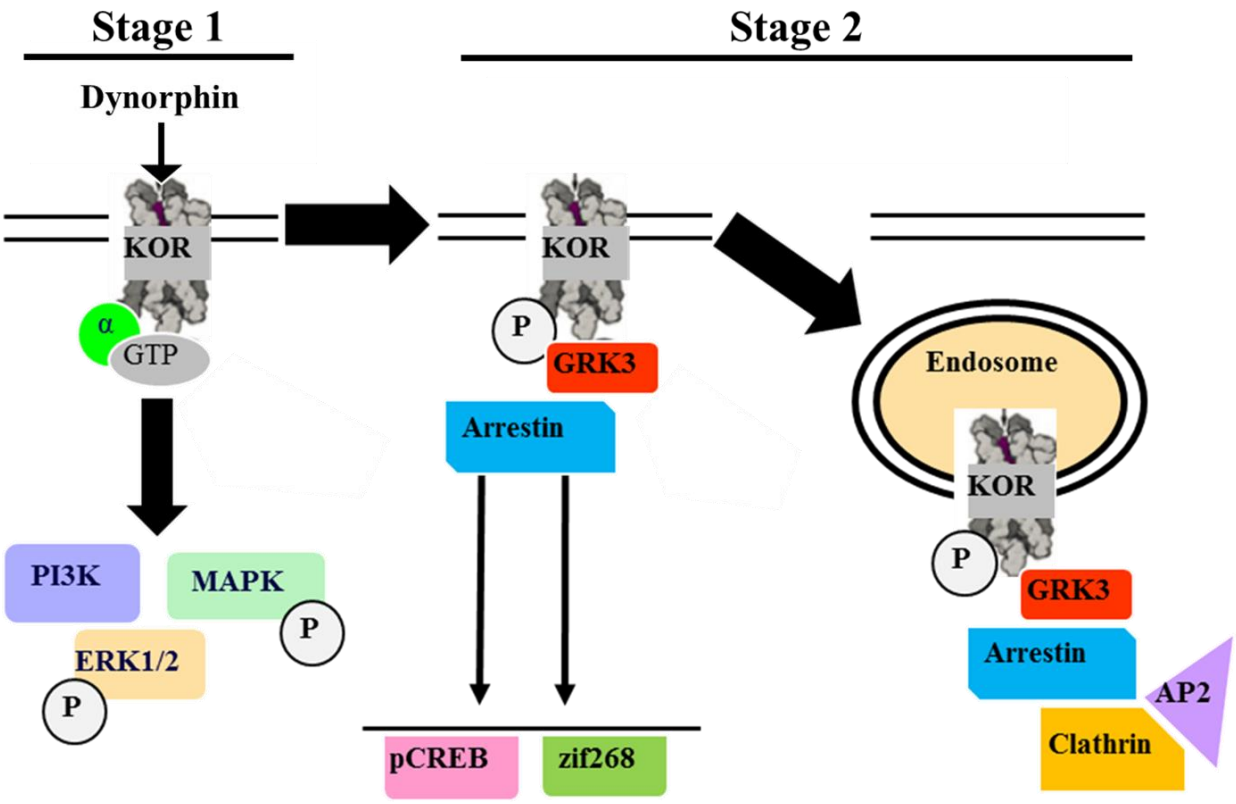

Figure 2: Summary diagram adapted from (Bruchas and Chavkin, 2010) outlining the different stages of KOR activation. Stage 1 ligand-signaled activation utilizes G-proteins to activate intracellular kinases such as P13K, MAPK, and ERK1/2. Stage 2 utilizes GRK3 to activate arrestin and downstream transcription factors pCREB and Zif268. At the end of Stage 2 KOR expression is downregulated via arrestin-dependent internalization.

Chapter 2 - Opioid Antagonism Impairs Acquisition of Forebrain-Dependent Trace-Associative Learning; an Eyeblink Conditioning Analysis[1]

Abstract

While the opioid system is predominantly known for its properties governing nociception, it has also been found to play a role in learning and memory. Opioid involvement in task acquisition and retention has been examined using various associative paradigms. These analyses have demonstrated that depending upon the associative paradigm and timing of opioid modulation relative to the task, it can either impair acquisition or facilitate memory consolidation. However, opioid involvement in forebrain-dependent trace-associative learning paradigms has never been examined. In associative paradigms, a subject learns to associate two stimuli while in trace paradigms the two stimuli are separated in time, which is thought to increase task difficulty due to utilization of forebrain structures. The current analysis utilized the trace paradigm whisker-trace-eyeblink (WTEB) conditioning with a trace interval of 250 ms, in conjunction with pre- and post-training opioid inhibition with naloxone, a well-characterized nonspecific opioid antagonist. Naloxone administration prior to training (pre-training) was found to significantly impair acquisition of the WTEB association; however, administration following training (post-training) did not significantly differ from saline controls. These findings demonstrate that opioid inhibition impairs acquisition of forebrain-dependent trace-associations, further suggesting that opioid activation plays a modulatory role in trace-acquisition. Prior behavioral analyses have suggested that hippocampal μ-opioid receptors are most likely facilitating this effect; however,

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subsequent analyses will be needed to determine the specific brain region(s) and opioid receptor subtype(s) mediating this effect.

Introduction

Associative learning paradigms are some of the oldest and most extensively used paradigms for examination of behavioral and biochemical mechanisms underlying learning and memory. In associative learning, a neutral conditioned stimulus (CS) is paired with a salient unconditioned stimulus (US) eliciting an unconditioned response (UR). After repeated CS-US pairings, the CS predicts the onset of the US, thus eliciting a learned conditioned response (CR). In delay conditioning the CS is presented and co-terminates with the US. This form of conditioning is forebrain independent, in that removal of the hippocampus or neocortex does not impair acquisition (Theios and Brelsford, 1966, Norman et al., 1977, Oakley and Russell, 1977, Mauk and Thompson, 1987). Rather, delay conditioning is dependent upon brainstem and cerebellar processing (Clark et al., 1984, Mauk and Thompson, 1987). In trace-conditioning, the CS and US are temporally separated by a stimulus-free trace interval, which recruits higher brain regions. For example, pre-training hippocampal or neocortical lesions impair acquisition of trace-eyeblink associations (Solomon et al., 1986, Moyer et al., 1990, Kim et al., 1995, Weiss et al., 1999, Takehara et al., 2002, Galvez et al., 2007). Furthermore, anatomical and biochemical analyses have demonstrated various forms of plasticity in both the hippocampus and neocortex during and following trace eyeblink conditioning (Thompson et al., 1996, Power et al., 1997, Moyer et al., 2000, Gierdalski et al., 2001, Galvez et al., 2006, Gruart et al., 2012, Chau et al., 2013). These and other analyses have provided much insight into the underlying mechanism for acquisition of trace-associations.

Behavioral analyses from other paradigms have further suggested that the opioid system is intimately involved in learning and memory. Pharmacologically inhibiting or genetically removing opioid receptors has been shown to impair acquisition on various behavioral paradigms, such as Morris Water Maze and 8-arm radial arm maze (Jamot et al., 2003, Jang et al., 2003, Sanders et al., 2005). Furthermore, studies using paradigms which are more typically viewed as delay-associative, such as shuttle-avoidance, extinction, and cued fear conditioning, have also demonstrated that opioid inhibition both before and after training impairs acquisition (Izquierdo et al., 1980, Messing et al., 1989, McNally et al., 2004, Meilandt et al., 2004, Kim and Richardson, 2009). Interestingly, μ-opioid activation has been found to significantly retard acquisition of delay-eyeblink conditioning in rabbits (Aloyo et al., 1993). However, opioid inhibition prior to delay-eyeblink training in rabbits showed no effect on acquisition but rather significantly facilitated extinction (Hernandez and Powell, 1983). These and other analyses have strongly suggested a role for the opioid system in acquisition of various learning tasks; however, opioid involvement in acquisition of neocortical dependent trace-associative paradigms has not been examined.

One trace-associative paradigm used in laboratory analyses of learning and memory is trace-eyeblink conditioning. In trace-eyeblink conditioning, a subject learns to associate a CS (e.g. sensory stimulation such as light, tone, or in rodents, whisker deflection) with a US that causes the subject to blink. Eyeblink conditioning is one of the few behavioral paradigms that is routinely used in various species, including humans, greatly facilitating the translational ability of these and subsequent findings across species. The following study used the opioid antagonist, naloxone, in conjunction with the well-established 250 ms trace-associative paradigm, whisker-

trace eyeblink (WTEB), to determine if the opioid system plays a modulatory role in acquisition of forebrain-dependent trace-associative learning.

Materials/Methods

Animals

Thirty-six 3 to 6 month-old male C57BL/6J mice were individually housed on a 12-hour light-dark schedule with lights on at 0700. Mice were provided access to food and water ad-libitum. All procedures performed were reviewed and approved by the University of Illinois Animal Care and Use Committee.

Surgery

The surgical procedure was performed as previously described (Galvez et al., 2009). Briefly, mice were anesthetized with a ketamine (1 mg/kg IP) xylazine (6 mg/kg IP) cocktail. Once anesthetized, a plastic strip connector with two Teflon-coated stainless steel wires and one uncoated ground wire was affixed to the head (headgear). The two coated wires were fed through the skin and left exposed at the periorbital region of the right eye. The ground wire was secured to a ground screw in the skull. Dental acrylic cement secured the headgear to the skull. All mice were given a minimum of five days to recover from surgery prior to WTEB training.

WTEB Procedure

Mice were placed into standard (12”x12”) laboratory cages in a sound-attenuated chamber. All procedures took place between 0900 and 1400. Mice were connected via their headgear to a tether that allowed free mobility while within the training cages. One day prior to testing, mice were habituated to the training cage and tether for 10 min. After habituation, mice were randomly assigned to receive either pre-training naloxone (n=10), post-training naloxone (n=10), pre-training saline (n=9), or post-training saline (n=7). At the time of training, the tether

was connected to a computer running a custom LabView program that delivered stimuli (whisker stimulation and periorbital shock), and acquired data (blink response and properties).

On each training day, mice were conditioned as previously described (Galvez et al., 2009). Briefly, mice were presented with a CS consisting of 250 ms whisker stimulation delivered via a custom whisker stimulator (Galvez et al., 2009), paired with a 100 ms periorbital shock US (0.1 to 0.5 mA periorbital square wave shock, 60 Hz, 0.5 ms pulses, Figure 3a, top). The shock intensity was tailored for each animal to generate a detectable blink response (Figure 3b). Mice were given 30 trials per day with a 15 to 30 s (mean of 20 s) inter-trial interval (ITI). An optic sensor that was attached to the tether was used to record closure of stimulated eyelid. A CR was defined as a 4-standard-deviation change in voltage from baseline occurring 20 ms prior to US onset (Figure 3a). Mice were trained with 30 trials per day for 8 days or until criterion, defined as 4 CRs out of 5 consecutive trials. The mean number of days for the combined saline groups to reach criterion was 3.70 days and all saline mice reached criterion by 6 days of training. A subset of the naloxone mice did not reach criterion while on the drug after 8 days of training. These mice were assigned a criterion day of eight; however, to ensure that they were capable of learning the association, drug administration was discontinued while continuing training. All of these mice reached criterion within 3 days of subsequent training while not receiving naloxone.

Drugs/Dosing

Mice were randomly assigned to either pre-training naloxone, post-training naloxone, pre-training saline or post-training saline conditions. On the day of training, mice were injected with naloxone (5 mg/kg IP; Sigma-Aldrich, St. Louis, MO) or saline either 8 min prior to (pre-training) or immediately following (post-training) WTEB conditioning. Prior associative learning

paradigms have demonstrated that 5 mg/kg of naloxone IP administered approximately 8 minutes prior to training impairs acquisition of fear conditioning extinction in young mice (Kim and Richardson, 2009). Additionally, lower doses given 5-10 minutes prior to training have been shown to exert similar effects in rats (Izquierdo et al., 1980, Messing et al., 1989). The half-life of naloxone in a mouse brain is approximately 30 minutes (Kishioka et al., 2013), suggesting that even with an 8 minute pre-training injection, the mice will be under the influence of the drug during the entire training session. Training takes approximately 20 minutes. All mice received one injection per day on each day of training.

Statistics

One-way ANOVA was used to analyze days to criterion and blink properties of conditioned mice. Additionally, a two-way ANOVA with repeated measures with degrees of freedom adjustment via Kenward-Rogers, was used to analyze percent CR task acquisition across successive days. Ad-hoc comparisons were analyzed in SAS 9.3, with multiple comparisons corrected using a Bonferroni correction.

Results

An overall ANOVA demonstrated that pre-training naloxone significantly inhibited acquisition of WTEB (Figure 4a). Planned contrasts demonstrated a significant difference in the number of days to reach criterion between pre-training naloxone and pre-training saline [$F(1,32) = 11.73$, $p = 0.0017$], post-training saline [$F(1,32) = 12.67$, $p = 0.0012$], and post-training naloxone [$F(1,32) = 12.97$, $p = 0.0011$]. Pre-training naloxone mice reached criterion in an average of 5.90 days (± 0.4712). Post-training naloxone achieved criterion in an average of 3.50 days (± 0.4712). Pre-training saline mice took an average of 3.56 days (± 0.4967) to reach criterion. Post-training saline mice took an average of 3.29 days (± 0.5632; Figure 4). No

significant differences were detected between post-training naloxone and either saline group. Additionally, the saline groups did not significantly differ from each other. Repeated measures ANOVA with Kenward-Rogers degrees of freedom adjustment found that all groups exhibited a significant within-subject increase in percent CR across days leading up to criterion (C-3, C-2, C-1, C) [$F(4, 90.7) = 18.25$, $p < 0.0001$]; however, there was no significant group by criterion-day interaction. These findings suggest that all animals exhibited similar acquisition curves at different time points (Figure 4b). Pre-training naloxone mice that did not reach criterion after 8 days (n = 3), reached criterion within 3 days after discontinuing the drug and did not significantly differ in their criterion-day percent CR from the other groups.

Due to well-known effects of opioids on pain modulation and possible effects on altering blink properties, training shock intensities and blink properties were also examined. There were no significant differences on the first day of training for the threshold shock intensity (Figure 3b) or any blink properties (UR onset, UR peak, UR peak time, or UR max slope) suggesting that the dose of naloxone used did not alter blink induction or sensitivity (data not shown). Further analyses of conditioned blink properties on the last day of training (criterion) also demonstrated no significant differences in CR onset, CR peak time or CR max slope (data not shown). Interestingly, post-naloxone mice exhibited significantly larger CR peaks than any of the other groups [$F(3, 29) = 7.48$, $p = 0.0007$], including pre- and post-training saline mice; however, no other significant differences were observed. These findings further suggest that the concentration of naloxone used did not have an effect on blink properties.

Discussion

Prior analyses have suggested that the opioid system plays a modulatory role in acquisition of associative learning tasks; however, opioid involvement in acquisition of

forebrain-dependent trace-associative paradigms has never been explored. The current study utilized WTEB conditioning to examine opioid involvement in acquisition of trace-associations. Our analyses demonstrated that pre-training opioid antagonism significantly impairs acquisition of the trace-association. These findings are consistent with prior behavioral analyses demonstrating impaired task acquisition with opioid inhibition (Messing et al., 1989, McNally et al., 2004, Meilandt et al., 2004, Kim and Richardson, 2009). Opioid inhibition has also been shown to facilitate performance when administered prior to testing when the subject is trained in the absence of any drug (Castellano, 1975, Izquierdo, 1979, Izquierdo et al., 1980, Canli et al., 1990, Ilyutchenok and Dubrovina, 1995, Zhu et al., 2011, Zarrindast et al., 2013); however, opioid-enhanced memory recall was not explored in the current analyses. These studies, along with the current analyses suggest that the opioid system plays an indiscriminate role in modulating behavioral acquisition of both trace- and non-trace associations.

The opioid system has three primary receptor subtypes (μ, κ, and δ), as well as a more recently discovered subtype orphanin FQ (Feng et al., 2012). The μ-opioid receptor has been well documented in causing significant and potent analgesia (Tsou and Jang, 1964). Activation of κ-opioid receptors are thought to mediate analgesia, sedation, and dysphoria (Von Voigtlander and Lewis, 1982); while activation of δ-opioid receptors play a modulatory role in, and induce analgesia (although to a lesser extent than μ-opioid receptors) (Ananthan, 2006). Naloxone, the opioid antagonist used in the current analyses, has a very strong affinity to all receptor subtypes, thus we are unable to make any specific claims as to the receptor subtype mediating the observed behavioral impairment. However, as discussed below, μ-opioid inhibition is the most likely cause for the current findings.

In addition to modulating behavior, opioid modulation (specifically via naloxone administration) has been shown to alter nociception. For example, naloxone has been shown to cause sensitization to heat pain (Jacob et al., 1974). Naloxone has also been shown to have no effect on pain detection for an electric foot shock (Goldstein et al., 1976). Altered nociception in the current analysis could impair blink induction and blink responses. Consistent with Goldstein et al. (1976), our analysis of the shock intensity threshold required to induce a blink did not significantly differ across any of the groups (Figure 3b). Furthermore, we did not detect any significant differences in UR blink properties and most CR blink properties, suggesting that naloxone, at the dose used in the current analyses, did not significantly alter the blink response. These findings collectively suggest that altered nociceptive properties are not a probable explanation for the observed opioid induced impaired WTEB acquisition.

The current study further demonstrated that post-training opioid inhibition, in contrast to pre-training inhibition, does not alter WTEB acquisition. These findings are in contrast to prior associative analyses demonstrating that post-training opioid antagonism modulates subsequent behavioral performance (McGaugh et al., 1988, Introini-Collison et al., 1989, Wassum et al., 2011, Zarrindast et al., 2013). Although these findings appear to be inconsistent with the current analyses, post-training memory modulation is known to be time sensitive, with increasing delays in time diminishing the effect of the manipulation. Each WTEB training session takes approximately 20 min to complete, thus our post-training injections were administered approximately 25 min after the start of the training session. If the opioid system plays a time sensitive role in task acquisition, the 25 min post-training injection could be beyond that critical window, impairing naloxone's ability to modulate acquisition of the association.

The current analyses demonstrate that opioid inhibition impairs trace-associative learning; however, based upon the current analyses, the specific brain region(s) mediating this effect is unknown. Prior whisker-trace associative analyses have implicated a potential role for primary somatosensory cortex (S1) in trace-acquisition. Pre-training S1 lesions completely impair WTEB acquisition (Galvez et al., 2007). WTEB conditioning has also been shown to cause metabolic and synaptic plasticity in layer IV of SI (Gierdalski et al., 2001, Siucinska and Kossut, 2004, Galvez et al., 2006, Siucinska, 2006, Galvez et al., 2011b, Chau et al., 2013). These studies have demonstrated a prominent neocortical role for WTEB acquisition, although, neocortical opioid involvement in memory modulation has not been investigated.

Prior behavioral analyses have suggested that opioid modulation in the amygdala can alter task consolidation. For instance, post-training intra-amygdala naloxone administration has been shown to facilitate performance for inhibitory avoidance (Gallagher and Kapp, 1978, McGaugh et al., 1988, Introini-Collison et al., 1989). These amygdalar effects are consistent with findings demonstrating that under certain conditions opioid agonists inhibit, while antagonists facilitate retention (Castellano, 1975, Izquierdo, 1979, Izquierdo et al., 1980, Canli et al., 1990, Ilyutchenok and Dubrovina, 1995, Zhu et al., 2011, Zarrindast et al., 2013). It should be noted that amygdala analyses have primarily shown this effect in post-training dosing schedules (Gallagher and Kapp, 1978, McGaugh et al., 1988, Introini-Collison et al., 1989). Although associative learning theories have suggested a role for the amygdala in focusing a subject's attention towards the CS (Chau and Galvez, 2012), amygdala involvement in trace-eyeblink conditioning, especially amygdala opioid involvement, has not been extensively explored. Based upon the fact that prior amygdala opioid associative analyses have demonstrated opposite effects

to those observed in the current analysis, we believe amygdala opioid receptors to be an unlikely site of action for our trace analyses.

Opioid behavioral analyses have also demonstrated that modulation of the μ-opioid receptor in the hippocampus can alter learning and memory. For example, μ-opioid knockout mice have been shown to exhibit decreased CA3 LTP (Jamot et al., 2003). Furthermore, CA3 μ-opioid inhibition blocks retention for the Morris Water Maze and inhibits reversal learning (Meilandt et al., 2004). These analyses suggest that hippocampal μ-opioid receptors play a role in acquisition and retention for various paradigms. Based upon the fact that the hippocampus is required for acquisition of trace-associations (Solomon et al., 1986, Moyer et al., 1990, Weiss et al., 1999, Takehara et al., 2002), these findings collectively suggest that hippocampal μ-opioid receptors are a likely site of action for the observed opioid effects.

Conclusion

The current analyses set out to determine if the opioid system plays a role in acquisition of forebrain-dependent trace-associations. Using the opioid antagonist naloxone, we determined that systemic opioid inhibition impairs trace-acquisition, consistent with that observed following other non-trace associative paradigms (Izquierdo et al., 1980, Messing et al., 1989, McNally et al., 2004, Meilandt et al., 2004, Kim and Richardson, 2009). Although subsequent analyses will be needed to determine the specific site of action for the opioid-dependent behavioral modulation, prior analyses suggest that the observed impairment in trace-acquisition is due to inhibition of hippocampal μ-opioid receptors; however, other brain regions and opioid receptor subtypes have not been extensively explored.

Figures

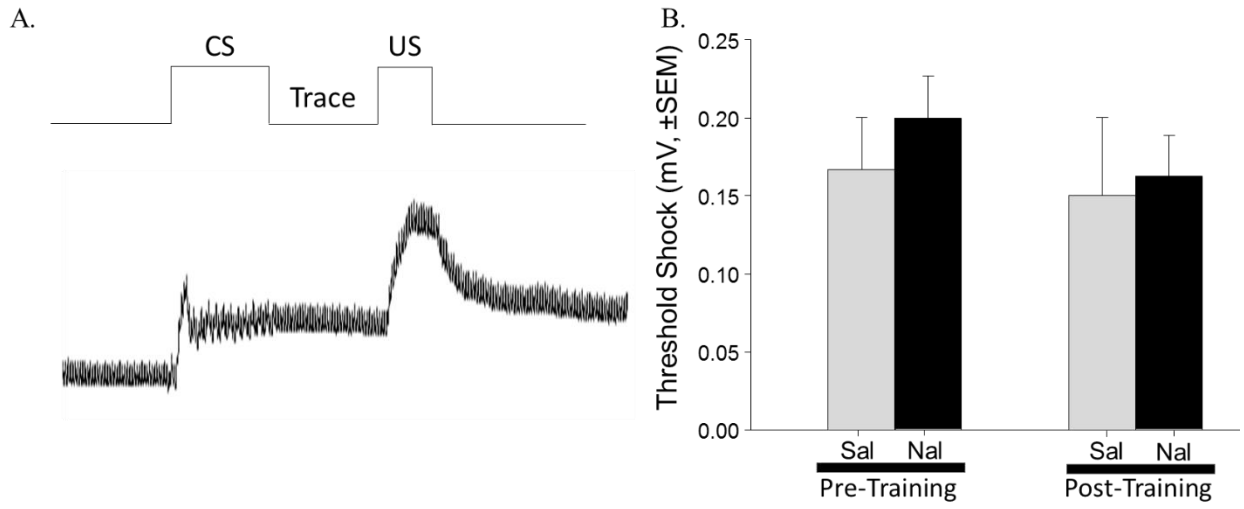

Figure 3: Schematic of conditioning paradigms and shock intensity levels. A) Top: Schematic illustrating the conditioned stimulus (CS; 250 ms), trace interval (250 ms), and unconditioned stimulus (US; 100 ms) administration. Bottom: A typical blink response exhibiting a conditioned response (CR) at the onset of the CS, and an unconditioned response (UR) at the onset of the US, during training. B) Mean shock intensity required to cause the mouse to blink. There was no significant difference across groups suggesting that opioid antagonism did not significantly alter blink induction. SEM = standard error of the mean; Sal = Saline; Nal = Naloxone.

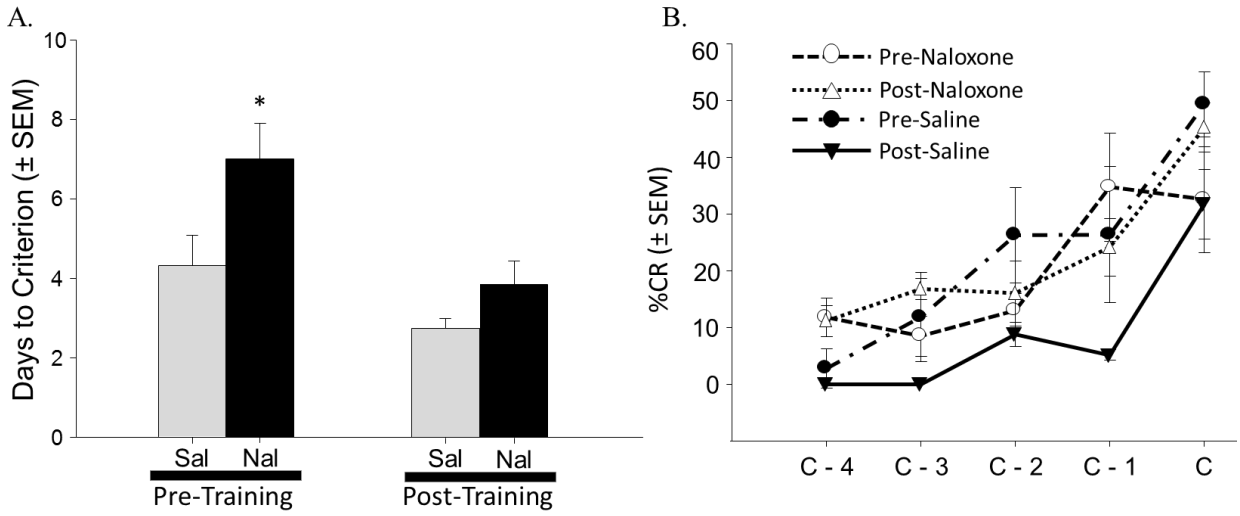

Figure 4: Opioid antagonism significantly impairs acquisition for WTEB conditioning. A) Mean days to criterion by group. Pre-training naloxone mice took significantly longer to reach criterion than all other groups. The effect was not observed for post-training naloxone, pre- nor post-training saline. B) Percent conditioned response (% CR) over 4 days leading to criterion (C = criterion, C-1 = one day before criterion, C-2 = two days before criterion, C-3 = three days before criterion, C-4 = four days before criterion). SEM = standard error of the mean; Sal = Saline; Nal = Naloxone (* = $p < 0.05$).

Chapter 3 - Kappa-Opioid Antagonism Impairs Forebrain-Dependent Associative Learning; a Trace Eyeblink Conditioning Study[2]

Abstract

The opioid receptor system is well known for its relationship to painful stimuli but has also been discovered to have a role in acquisition and consolidation of associative memories. Most opioid receptor specific studies have focused on, and attributed these findings to modulation of the mu-opioid receptor (MOR); however, some studies have suggested that the kappa-opioid receptor (KOR) also plays in role in memory modulation. The following study set out to determine KOR involvement in acquisition for forebrain-dependent associations. Using the forebrain-dependent associative task whisker-trace eyeblink conditioning (WTEB), the current study demonstrated that KOR inhibition via NorBNI (10 mg/kg) significantly delayed acquisition. To explore the brain region mediating these NorBNI-induced learning impairments, subsequent experiments focused on primary somatosensory cortex (S1). S1 plays a pivotal role in the acquisition of WTEB with lesions either before or after conditioning inhibiting acquisition or retrieval respectively. NorBNI (10µg or 20µg) in S1 was found to significantly delay acquisition, similar to that observed following systemic injections. In support of these findings, studies have suggested a role for dynorphin (KOR's endogenous ligand) expressing GABAergic interneurons in cortical processing of whisker information. Although, additional studies will be required to determine the specific mechanism for KOR and these GABAergic interneurons; these findings

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strongly support previous studies suggesting KOR involvement in learning mechanisms, while elucidating an unexplored neocortical learning mechanism.

Introduction

The opioid system has been shown to play a modulatory role in acquisition of learned associations. General opioid inhibition impairs acquisition for both associative and non-associative behavioral tasks such as shuttle avoidance, classical conditioning, operant conditioning, and water maze (Izquierdo et al., 1980, Messing et al., 1989, McNally et al., 2004, Meilandt et al., 2004, Kim and Richardson, 2009, Loh and Galvez, 2014). In exploring the specific opioid receptor subtype mediating these effects, many have focused on the Mu-opioid receptor (MOR). Although studies have found that MOR can alter acquisition for many of these paradigms (Jamot et al., 2003, Jang et al., 2003, Meilandt et al., 2004, Sanders et al., 2005), studies have recently suggested that the kappa-opioid receptor (KOR) also plays a role in modulating learning and memory.

In exploring the role of KOR in modulating learning, a few studies have emerged suggesting that KOR can alter learning. For example, application of the KOR specific endogenous ligand dynorphin A-(1-13) has protective effects against scopolamine (Itoh et al., 1993b) and ischemia induced (Itoh et al., 1993c) Y-maze spontaneous alternation deficits. Elevating hippocampal dynorphin levels also causes deficits in water maze performance (Jiang et al., 1989, Sandin et al., 1998); while KOR knockout mice were found to exhibit fewer errors on the Morris Water Maze across several days of training compared to wild-type controls (Jamot et al., 2003). In addition to these findings, KOR antagonists have been shown to inhibit acquisition of contextual fear conditioning in rats (Fanselow et al., 1991) and delay-eyeblink conditioning in rabbits (Schindler et al., 1986, Schindler et al., 1987). Similarly, systemic or prefrontal KOR

agonist treatment effectively blocked alcohol reinstatement or withdrawal induced conditioned place aversion in rats respectively (Marchant et al., 2010, Kelsey et al., 2015). These studies collectively suggest that KOR modulates various types of learning; however, a clear role for KOR involvement in neocortical-dependent associative learning has not been deduced.

To examine the role of KOR in acquisition of trace associations, the following study used the well-characterized associative paradigm, trace-eyeblink conditioning. In trace-eyeblink conditioning the subject learns to associate a neutral conditioned stimulus (CS) with a salient unconditioned stimulus (US) that induces an eyeblink. This form of conditioning has been used in various species and is dependent upon forebrain structures such as the hippocampus and neocortex (Solomon et al., 1986, Moyer et al., 1990, Kim et al., 1995, Weiss et al., 1999, Takehara et al., 2002, Galvez et al., 2007). Specifically, lesions to primary somatosensory neocortex (S1) prior to conditioning completely inhibit acquisition for whisker-trace-eyeblink conditioning (WTEB), a form of trace-eyeblink conditioning that uses whisker stimulation as the conditioned stimulus (Galvez et al., 2007). Additionally, this form of conditioning induces neuronal anatomical plasticity in S1 that is believed to mediate consolidation of the association (Chau et al., 2014a).

The current study utilized the uniquely long acting KOR antagonist nor-binaltorphimine (NorBNI, CID 5480230) to study the role of KOR in WTEB. NorBNI is a unique drug that antagonizes KOR function long after it is no longer detectable in plasma or brain. Initially NorBNI has an affinity for both MOR and KOR that after about 4 hours becomes selective for only KOR (Munro et al., 2012). Biochemical studies have suggested that the mechanism of this action is likely to be phosphorylation of the c-Jun N-terminal Kinase (JNK) (Bruchas et al., 2007); however, the exact mechanism with which JNK is capable of inhibiting KOR remains

unknown. Studies have suggested that there is a yet-to-be discovered protein dubbed JNK Modulated Regulator (JMR) that is activated by phospho-JNK, effectively antagonizing KOR activation for up to 28 days (Figure 5) (Horan et al., 1992, Bruchas et al., 2007, Munro et al., 2012). To ensure only antagonism of KOR, NorBNI was injected 24 hours before the first day of training, directly following habituation.

Materials/Methods

Animals

Fifty-three 3 to 6-month-old male C57BL/6J mice were housed individually in standard (12”x12”x12”) laboratory cages. A 12-hour light-dark schedule (lights on at 0700) was used. Housing rooms were temperature controlled (~21º C), and mice were provided ad-libitum access to food and water. All procedures performed were reviewed and approved by the University of Illinois Animal Care and Use Committee. All efforts were made to minimize animal suffering and utilize the fewest amount of animals possible.

Surgery

Surgeries were performed as previously described (Galvez et al., 2009). Mice were placed under anesthesia using a ketamine (1mg/kg i.p.) and xylazine (6mg/kg i.p.) cocktail. Once anesthetized, a ‘headgear’ consisting of a plastic strip connector with two Teflon-coated stainless steel wires and one uncoated ground wire was securely attached to the head. The Teflon-coated wires were fed under the skin into the periorbital region of the eye and left exposed, while the ground wire was securely fastened to a ground screw in the skull. The entire apparatus was then secured and affixed to the skull using dental acrylic cement. All mice were given at minimum seven days to recover from surgery prior to WTEB training.

Intracranial surgeries

Twenty-two of the mice also had a 26-gauge stainless steel guide cannula (4 mm in length; PlasticsOne, Roanoke, VA) inserted into S1 contralateral to the whisker stimulator (-0.8 mm AP, 3 mm ML, and -0.5 mm DV to bregma) (Franklin, 2008). The guide cannula was fixed to the skull and headgear with dental cement. Upon completion of the surgery an obdurator was screwed into the guide cannula and remained in place until the mouse was injected.

WTEB Procedure

Mice were placed into standard laboratory cages (12”x12”x12”) in a sound attenuated chamber. All WTEB training took place between the hours of 0900 and 1400. The headgear described in section 2.2 was then connected to a tether that was connected to a computer running a custom program written in LabView that delivered both stimuli (whisker stimulation and shock), and acquired blink properties. The tether provided freedom of mobility during the training procedures. One day prior to training onset, mice were habituated to the tether and training chamber for 10 minutes. On training days, mice were conditioned as previously described (Galvez et al., 2009). A presentation of the CS (250 ms whisker stimulation) was paired with a US (100 ms periorbital shock, 0.1-0.5 mA periorbital square wave shock, 60Hz, 0.5 ms pulses). The US shock intensity was tailored to each mouse to generate a detectable blink response with minimal voltage. The CS and the US were separated by a 250 ms stimulus-free trace interval (Figure 6). Mice were presented with the association 30 times per day with an inter-trial interval between 15 and 25 s (mean of 20 s). An optic sensor attached to the tether was used to record blink responses. A conditioned response (CR) was defined as 4-standard-deviation change in voltage from baseline occurring after CS onset and within 20 ms prior to US onset (Figure 6). These settings are consistent with that used by other laboratories conducting eyeblink

conditioning analyses (Moyer et al., 1990, Tseng et al., 2004, Weiss and Disterhoft, 2011). Mice received 30 paired presentations of the stimuli per day until criterion was achieved, defined as 4 CRs out of 5 consecutive trials. Previous studies from our laboratory have demonstrated this criterion point to be consistent with acquisition of the trace association (Chau et al., 2013).

Drug/Dosing

Experiment 1: Following habituation mice were randomly assigned to receive one of four conditions: saline (n=8), or one of three doses of NorBNI: NOR2.5 (2.5 mg/kg; n=8), NOR5.0 (5.0 mg/kg; n=8), and NOR10.0 (10 mg/kg; n=7); (Sigma-Aldrich, St. Louis, MO). Mice received a single IP injection 24 hours prior to the start of WTEB training.

Experiment 2: Following habituation twenty-two mice were divided into three groups in which they received a unilateral intracranial injection of either 10µg (n=8) or 20µg (n=8) NorBNI, or saline (n=8) into S1 contralateral to whisker stimulation. The following doses have been shown to not affect pain sensitivity measured with a tail-flick assay (Bruchas et al., 2007, Munro et al., 2012). Furthermore, the lowest dose (10µg) has been shown via intracerebroventricular injections to inhibit acquisition for fear associations in rats (Fanselow et al., 1991). Intracranial injections were conducted at a flow rate of 0.5µl/minute for 2 minutes for a total volume of 1µl (Hamilton 25µl gastight syringe, Fisher Scientific; Legato 101 pump; KD scientific; Hollison, MA). After the injection, the needle was left in the cannula for 1 minute to ensure drug delivery was complete. The following day mice were WTEB conditioned as outlined above. All mice were trained to behavioral criterion of 4 blinks out of 5 CS-US pairings. Upon completion of experiment 2, mice were sacrificed and brains were sectioned at 30µm and nissl stained with pyronin-y to verify cannula placement.

Statistics

A one-way ANOVA was used to analyze days to criterion and blink properties of trained mice. Additionally, a two-way ANOVA with repeated measures and degrees of freedom adjusted via Kenward-Rogers was used to analyze percent CR across successive days. Pre-planned comparisons were analyzed in SAS 9.3, with Bonferroni correction for multiple comparisons.

Results

Experiment 1: Four mice were excluded from the analyses due to improper surgical placement of the optic sensor. Due to possible effects of peripheral kappa modulation on the mouse's ability to blink, blink properties were also examined. To avoid possible changes in blink properties due to learning (Chau et al., 2013), these analyses were conducted on the first day of training before significant learning had occurred. Examination of blink properties (UR Onset, UR peak, UR peak time, or UR max slope) on the first day of training demonstrated no significant differences between groups (Figure 7). Peripheral kappa modulation has also been shown to alter nociception (Kolesnikov et al., 1996); however, measurements of the threshold shock intensity required to induce a blink yielded no significant differences between groups (Data not shown). These data suggest that NorBNI, at the concentrations used, did not significantly alter the ability of the mice to blink.

After removal of the four mice, the remaining 27 mice (saline n=7; NOR2.5 n=8; NOR5.0 n=6; NOR10.0 n=6) demonstrated an overall ANOVA with a significant effect of drug on the number of days to criterion [$F(3,23) = 4.45$, $p = 0.013$] (Figure 8A). Saline mice acquired the task in an average of 2.57 days (± 0.37), NOR2.5 took an average of 16% longer (3.00 days ± 0.35), NOR5.0 took an average of 30% longer (3.33 days ± 0.40), and NOR10.0 took an average of 75% longer (4.50 days ± 0.40). Pre-planned comparisons demonstrated there to be a

significant difference between saline and the average of all NorBNI groups [$F(1, 23) = 5.69$, $p = 0.026$] (Figure 8A). Additionally, the highest dose was significantly different from saline [$t(23) = -3.50$, $p = 0.0019$], NOR2.5 [$t(23) = -2.81$, $p = 0.010$], and NOR5.0 [$t(23) = -2.04$, $p = 0.053$]. After normalizing the behavioral data to the day of criterion for each animal (C), a repeated measures ANOVA with Kenward-Rogers degrees of freedom adjustment demonstrated that all groups acquired the task across criterion-days (C-4, C-3, C-2, C-1, C) [$F(4, 49.3) = 39.21$, $p < .0001$] (Figure 8B). However, there was no significant effect of group or group by criterion-day interaction.

Experiment 2: Two mice [1 Saline & 1 NorBNI (20µg)] were dropped from the analysis due to post-surgical complications. Due to NorBNI effects on pain and nociception the shock threshold necessary for inducing a blink was also examined. Consistent with experiment 1 there was no significant difference in the shock threshold required for obtaining a blink between groups (data not shown). An overall ANOVA demonstrated a significant effect in the amount of time it took NorBNI dosed animals to learn compared to saline controls [$F(2,19) = 11.04$, $p = .0007$]. Mice that received 10µg of NorBNI into S1 took on average 90% longer to acquire the association (4.88 days ± 0.34), while mice that received 20µg of NorBNI into S1 took on average 67% longer to acquire the association (4.29 ± 0.37) days compared to saline mice (2.57 days ± 0.37) (Figure 9A). There was no significant difference between the 10µg and 20µg doses. Similar to experiment 1, behavioral data was normalized to day of criterion for each mouse and repeated measures ANOVA with Kenward-Rogers degrees of freedom adjustment was performed. The repeated measures ANOVA demonstrated that all groups acquired the task across criterion-days (C-4, C-3, C-2, C-1, C) [$F(4, 50.3) = 46.78$, $p < .0001$] (Figure 9B). However, similar to experiment 1, there was no significant effect of group or group by criterion-day interaction.

Discussion/Conclusion

Many opioid studies of learning and memory have largely ignored KOR; however, recent findings have begun to elucidate a role for KOR in learning and memory processes. The current study used both systemic and intra-cortical infusion of the KOR-specific antagonist NorBNI in conjunction with the trace-associative paradigm WTEB to discern the neocortical role of KOR in forebrain-dependent associative learning. NorBNI is a potent, long-acting and selective KOR antagonist that after approximately 4 hours following administration has a more than 100x affinity for KOR than other opioid receptor subtypes (Munro et al., 2012). Biochemical studies have demonstrated that although NorBNI is 99.9% removed from the system within 24 hours, it maintains a high level of KOR antagonism for up to 28 days (Munro et al., 2012). The current study found that NorBNI, when administered 24 hours prior to the onset of training so that the effects would be exclusively for KOR, inhibited WTEB acquisition (Figure 8).

To explore the specific brain region mediating this effect, KOR was blocked with NorBNI in S1. The S1 region of the neocortex is widely interconnected and plays both a direct and indirect role in the acquisition of WTEB associations. It has been shown to be reciprocally connected to the hippocampus, pontine nuclei, thalamus, secondary somatosensory cortex and the basal ganglia (See (Galvez et al., 2011b) for review). Due to the fact that S1 displays such high interconnectivity to areas active in trace eyeblink conditioning, as well as having displayed active plasticity and morphological changes upon WTEB training (Chau et al., 2013, Chau et al., 2014b), S1 was a prime candidate to investigate KOR modulation of learning. The current study demonstrated that NorBNI (10µg and 20µg) given 24 hours prior to WTEB training directly into S1 significantly impaired acquisition, similar to that observed following systemic NorBNI injections. This finding establishes a precedent for the involvement of KOR in S1 mediated

learning. Specifically, to the authors' knowledge, this is the first reported manipulation of somatosensory neocortical KOR modulation of learning. These findings, along with our systemic injections collectively suggest that neocortical KOR activation plays a prominent role in acquisition of forebrain-dependent associations.

Interestingly, our experiment 1 data strongly suggests a one-way dose dependent increase, while experiment 2 data suggests a plateau in the time it took mice to acquire the WTEB association. Many drug-learning studies have been shown to exhibit a dose dependent inverted U-shaped response curve (Roozendaal and McGaugh, 2011). Although prior NorBNI studies have utilized a similar dose range (Bruchas et al., 2007, Munro et al., 2012); if an inverted U-shaped curve is indeed expected with NorBNI, a wider dose range would be prudent in subsequent studies.

In addition to its involvement in learning processes, the peripheral opioid system also has a well-characterized role in perception of nociceptive stimuli that could alter acquisition for the WTEB association. However, the current study did not detect any significant differences in the shock intensity required to induce a blink or any of the blink properties across any of the groups for both experiments. These findings strongly suggest that NorBNI, at the concentrations used in the current study, did not significantly alter nociception or the ability to blink. However, the current study did not directly test the possibility that NorBNI altered whisker perception, or the CS signal, impairing acquisition for the trace association. We have previously shown that S1 lesions can significantly impair acquisition for the trace association (Galvez et al., 2007). While a possible hypothesis, the NorBNI doses used have been shown to not alter perception/detection for other sensory systems (Bruchas et al., 2007, Munro et al., 2012). Furthermore our analyses demonstrated that NorBNI did not have an effect on periorbital shock detection or any blink

properties. Although the current study cannot completely rule out NorBNI modulation of whisker detection, based upon the fact that the NorBNI doses used have been shown to not have an effect on other sensory systems (Bruchas et al., 2007, Munro et al., 2012), we believe this to be an unlikely explanation for the observed NorBNI induced learning impairing effects.

Our finding that blocking KOR impairs acquisition for forebrain-dependent associative learning is consistent with some KOR learning studies. For example, (Tejeda et al., 2013, Kelsey et al., 2015) have found that antagonizing KOR hinders acquisition for conditioned place aversion. Additionally, KOR activation via endogenous or exogenous agonists has been shown to have memory-protective effects following chemical or ischemia induced Y-maze spontaneous alternation deficits (Itoh et al., 1993a, b, c). While these studies support our current study, some have demonstrated opposing results. Stress-induced endogenous activation of KOR impairs novel object recognition (Carey et al., 2009), and KOR knockout mice exhibit slightly enhanced Morris Water Maze acquisition (Jamot et al., 2003). Although these studies seem to contradict in the specific role for KOR in mediating different forms of learning, they collectively demonstrate KOR involvement in learning and memory. Future studies will need to determine the specific mechanism(s) mediating these learning differences.

Interestingly our findings demonstrated that although NorBNI impaired acquisition for the trace association, the learning curves of the groups remained the same. This pattern of learning has also been observed following medial septal lesions (Berry and Thompson, 1979) and is consistent with a lengthening of phase 1 in Prokasy's two-phase model for learning classical conditioning (Prokasy et al., 1974). KOR activation has been shown to stimulate many associative learning-related mechanisms. Specifically, KOR activation phosphorylates ERK1/2 (extracellular signal-regulated kinases 1/2), PI3K (phosphatidylinositide 3-kinases), and MAPK

(mitogen-activated protein kinase) (Bruchas and Chavkin, 2010). These kinases are also activated following associative learning, and when blocked impair acquisition (Atkins et al., 1998, Shalin et al., 2004, Sui et al., 2008). Blocking KOR in the current study may have lengthened learning in phase 1 by impairing activation of these kinases in S1. Further studies will be needed to better elucidate the underlying mechanism by which KOR alters acquisition for the trace association.

One mechanism by which blocking KOR in S1 could have impaired acquisition for the trace association is through somatostatin containing GABAergic interneurons (SOM) in S1. Studies have shown that whisker associative learning increases GABA and GAD immunoreactivity (Siucinska et al., 1999, Gierdalski et al., 2001, Siucinska, 2006) and GABAergic synaptic transmission (Tokarski et al., 2007) within S1, demonstrating that whisker associative learning is associated with increased neuronal inhibition. However, whisker associative learning has also been associated with increased rather than decreased neuronal excitability (Bekisz et al., 2010). Many have speculated that this increased GABA activation with learning, results in a sharpening of the whisker response as observed during passive whisker stimulation (Miller et al., 2001, Sun et al., 2006). Although there are three prominent types of GABAergic interneurons in the rodent neocortex [parvalbumin (PV), SOM, and calretinin (CR) containing (Kosaka et al., 1987, Sohn et al., 2014) it is known that this learning mechanism is not mediated by PV interneurons, the only cell type currently explored (Siucinska and Kossut, 2006). SOM interneurons in S1 have not been explored for their role in this mechanism; however, anatomical studies have demonstrated that the vast majority of SOM interneurons co-express the precursor to KOR-ligand peptides, preprodynorphin (Sohn et al., 2014). These studies, along with the current KOR findings strongly suggest that SOM interneurons in S1 are contributing

towards these learning-induced processes, possibly via sharpening of the whisker response. As an interesting note, in the absence of whisker stimulation, SOM interneurons are depolarized, and when there is active or passive use of the whiskers, SOM interneurons become hyperpolarized (Gentet et al., 2012). The current study did not directly investigate these SOM interneurons, so future experiments will be required to further understand this interaction.

Conclusion

The current investigation set out to determine if KOR plays a role in acquisition of forebrain-dependent associations. These studies determined that systemic and intra-S1 KOR inhibition via NorBNI, impaired acquisition for WTEB conditioning. The finding offers further support for the role of the primary somatosensory cortex in WTEB. More importantly, these studies help establish a novel role for KOR in modulating neocortical learning mechanisms. To the authors' knowledge, this is the first study to examine the neocortical role of KOR in acquisition for forebrain-dependent associative learning. These findings not only solidify the role of KOR in modulating associative learning, but also broaden our understanding of opioid involvement in learning and memory while providing vital insight towards the molecular mechanism facilitating the learning process.

Figures

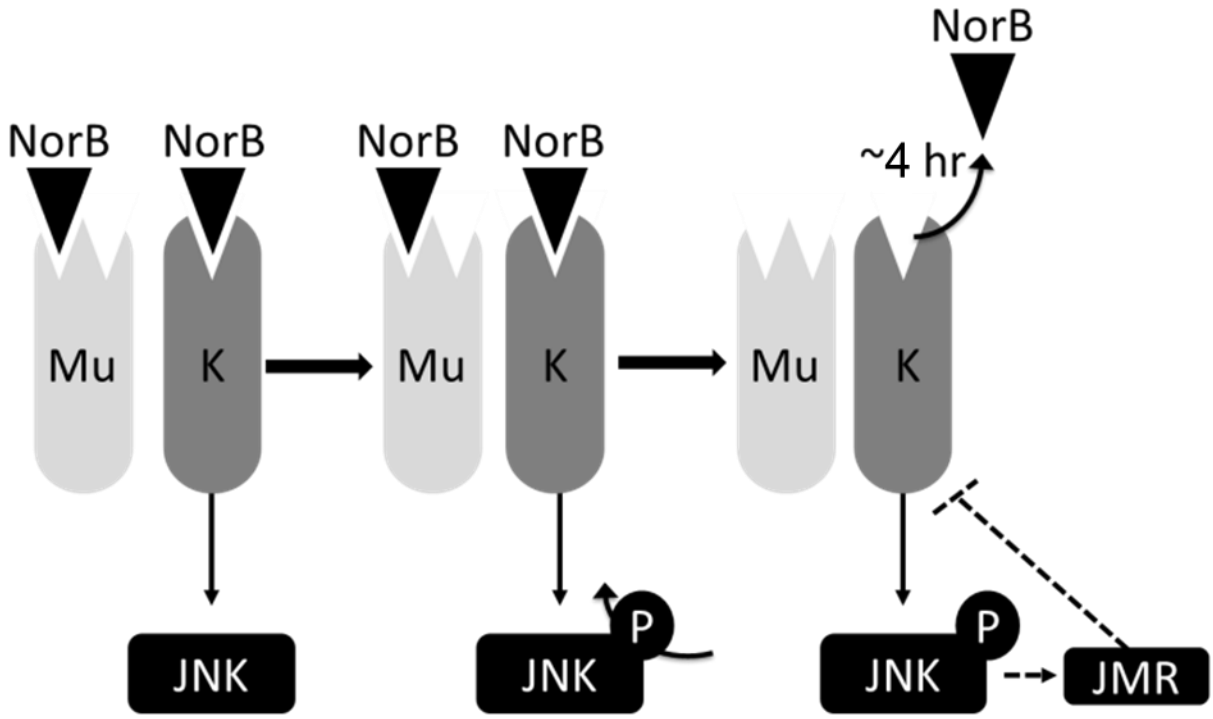

Figure 5: Schematic outlining the proposed mechanism for NorBNI mediated kappa receptor inhibition. NorBNI is a long acting, delayed-onset kappa opioid receptor antagonist. Upon initial application, NorBNI has affinity for, and antagonizes both mu and kappa opioid receptors. Upon binding to the kappa receptor, c-Jun N-terminal Kinase (JNK) is phosphorylated. After approximately 4 hours, NorBNI dissociates from both receptors and plasma/brain levels drop to negligible levels. Experimental literature has posited that the continued antagonism of kappa is due to the phosphor-JNK activation of a yet-to-be discovered protein, JNK modulation regulator (JMR) (Bruchas and Chavkin, 2010). NorB = Norbinaltirphimine; P = phosphorylated; K = Kappa Opioid Receptor; Mu = Mu Opioid Receptor.

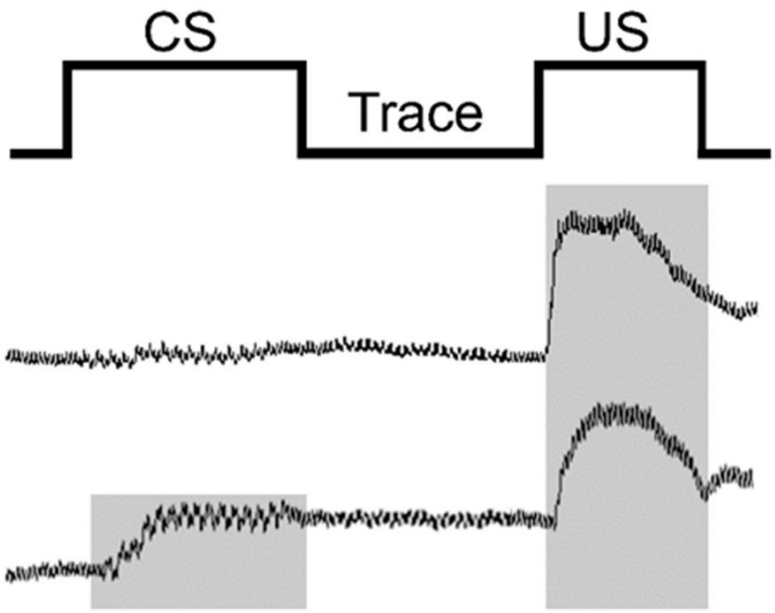

Figure 6: Schematic of stimuli and responses for WTEB conditioning paradigm. Top: Illustration of conditioning stimulus (CS; 250ms), trace interval (250 ms), and unconditioned stimulus (US; 100 ms). Middle: A typical blink response exhibiting a normal unconditioned response (UR) at the onset of the US (grey region). Bottom: A typical blink response demonstrating a conditioned response (CR) at the onset of the CS, as well as a normal UR at the onset of the US (grey regions).

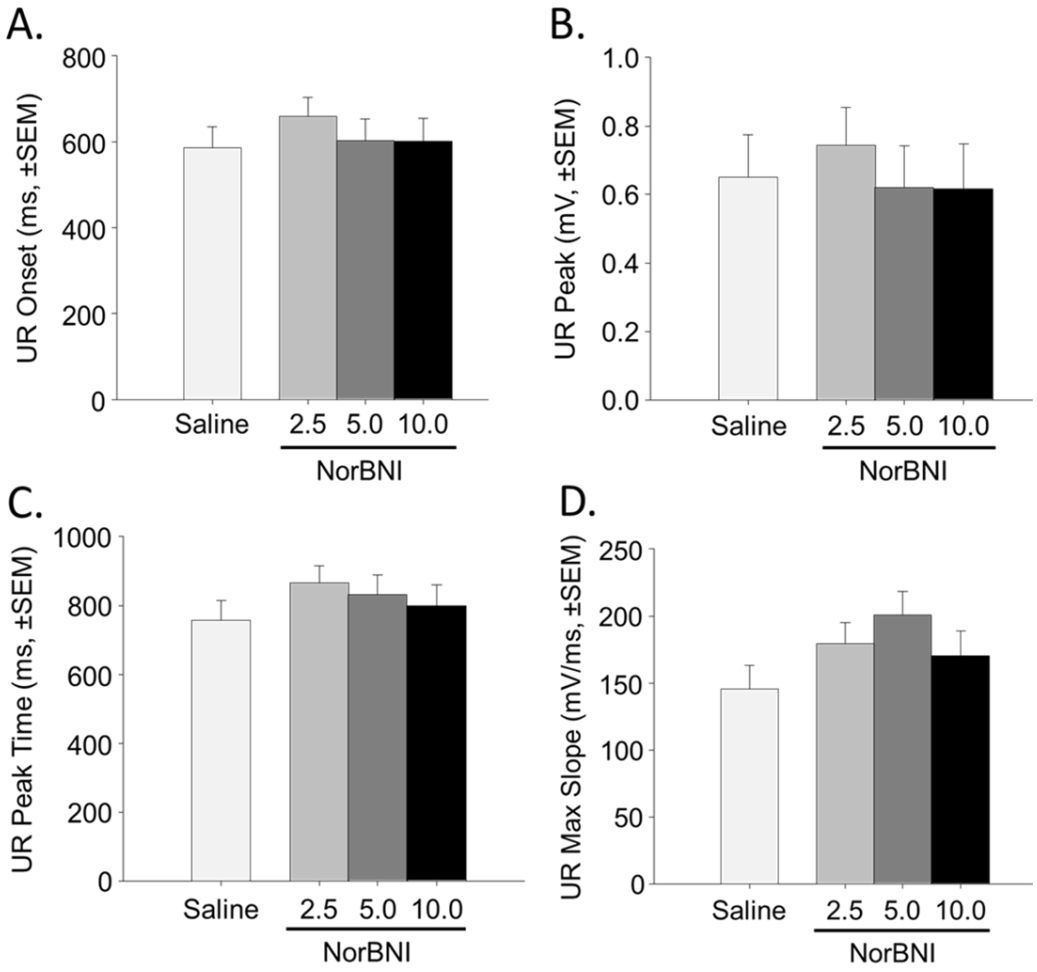

Figure 7: Kappa antagonism via NorBNI did not significantly alter blink properties. A) Mean time for mice in each group to display an unconditioned response (UR). B) Mean UR magnitude over baseline displayed by mice in each group. C) Mean time for mice in each group to reach their maximum blink response. D) Mean rate for mice in each group to display a blink. No significant differences were observed for any of the above measurements, suggesting that NorBNI, at the doses used, did not significantly alter their ability to blink. Blink properties were only examined on the first day of training to avoid learning effects. SEM = Standard error of the mean; NorBNI = Norbinaltorphimine.

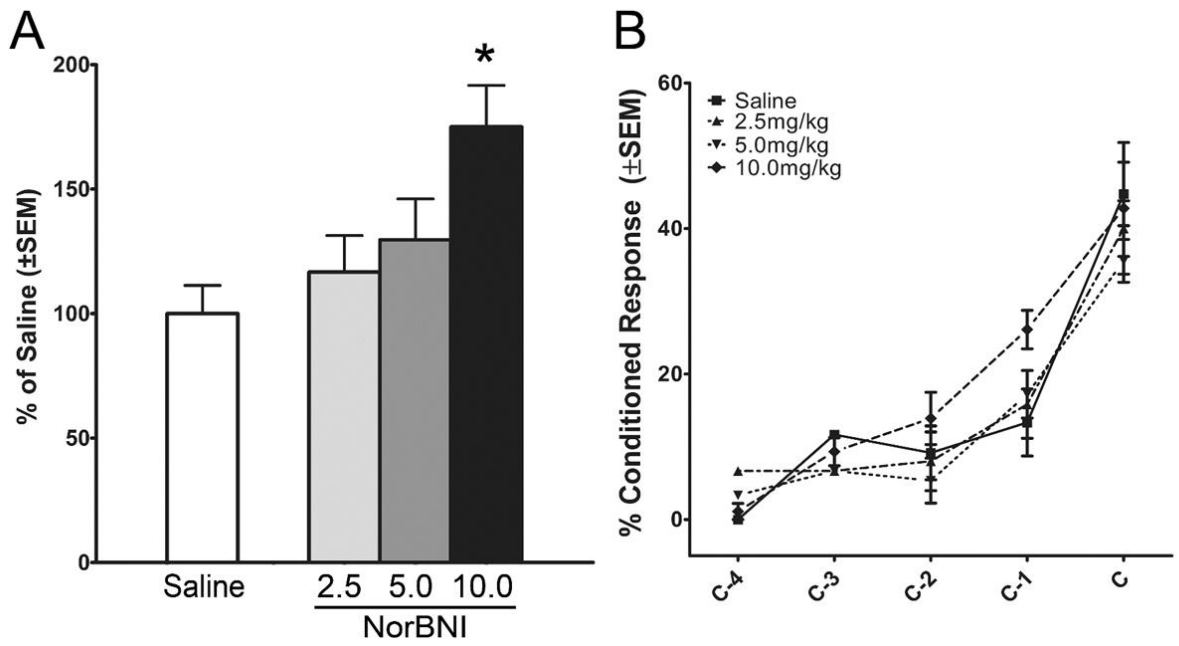

Figure 8: Kappa opioid antagonism significantly impairs acquisition of WTEB conditioning. A) Percent of time relative to saline that each group took to acquire the WTEB task. Mice that received 10mg/kg NorBNI took significantly longer to acquire the association than all other groups. B) Percent conditioned response (%CR) over 4 days leading to criterion (C = Criterion, C-1 = one day prior to criterion, C-2 = two days prior to criterion, C-3 = three days prior to criterion, C-4 = four days prior to criterion). Although mice acquired the association at different rates (A), the acquisition curves remained consistent (B). SEM = Standard error of the mean; NorBNI = Norbinaltorphimine (* = $p < 0.05$).

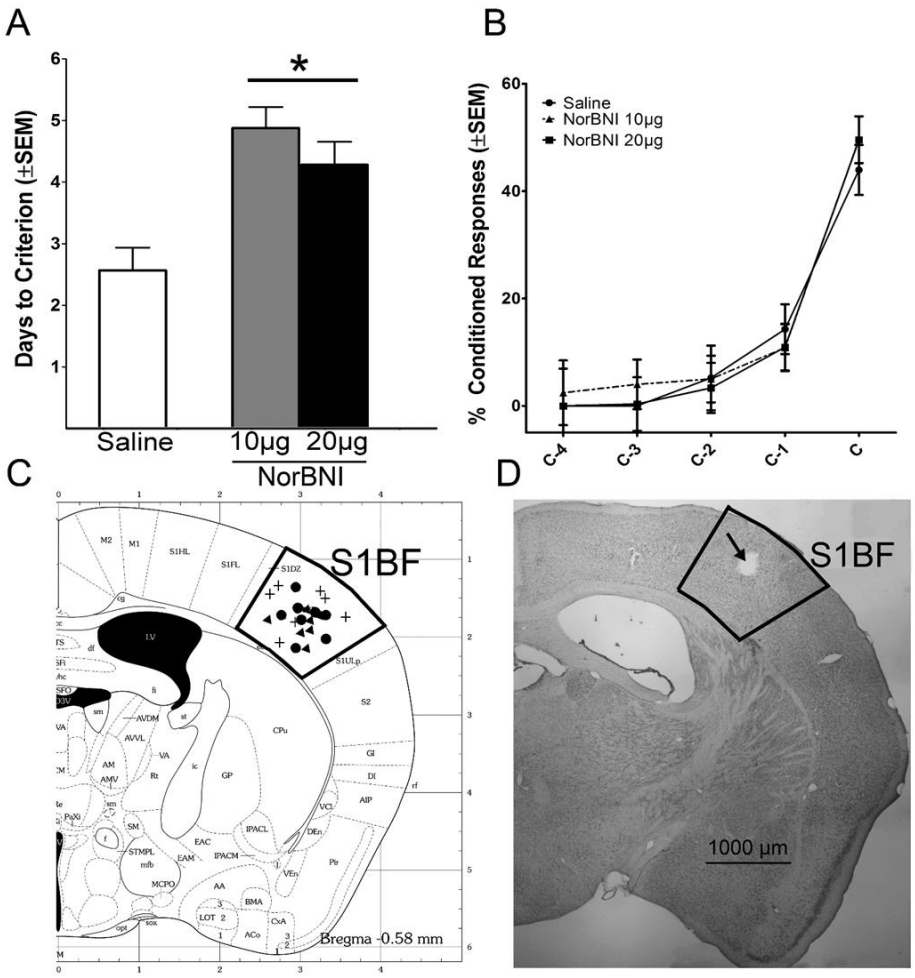

Figure 9: Kappa opioid antagonism within primary somatosensory cortex (S1) significantly impairs acquisition of WTEB conditioning. A) Mean number of days for each group to reach behavioral criterion. Mice that received 10µg or 20µg NorBNI took significantly longer to acquire the association than the saline group. B) Percent conditioned response (%CR) over 4 days leading to criterion. Although mice acquired the association at different points (A), the acquisition curves remained consistent (B). SEM = standard error of the mean. C) Mouse brain atlas showing localization of all 22 injection locations in S1 barrel sub field (S1BF) (Circles = 10µg, Triangles = 20µg, Pluses = Saline). D) Pyronine-Y stained representative section showing injection location in S1. NorBNI = Norbinaltoprhimine, (* = $p < 0.05$).

Chapter 4 - Antagonizing the Different Stages of Kappa Opioid Receptor Activation Selectively and Independently Attenuates Acquisition and Consolidation of Associative Memories[3]

Abstract

Previous work from our laboratory has shown that nonspecific kappa opioid receptor (KOR) antagonism in primary somatosensory cortex (S1) can inhibit acquisition for the forebrain-dependent associative task, Whisker-Trace Eyeblink conditioning (WTEB). Although studies have demonstrated that KOR activation can alter stimuli salience, our studies controlled for these factors, demonstrating that KOR also plays a role in facilitating learning. KOR has two distinct phases of activation followed by internalization/downregulation, that each independently activate kinases and transcription factors known to mediate task acquisition and memory consolidation respectively. The current study demonstrated that antagonism of the initial phase of KOR activation in S1 via local injections of the g-protein inhibitor, pertussis toxin (PTX), blocked initial WTEB acquisition without affecting retention of the association. In contrast, KOR late phase antagonism in S1 via local injections of the GRK3-specific antagonist, guanidinonaltrindole (GNTI), blocked retention of the WTEB association without affecting task acquisition. Consistent with the known mechanism for KOR activation, KOR protein expression in S1 was found to be decreased following WTEB training, further supporting the involvement of neocortical KOR activation with learning. Prior studies have shown that task acquisition and

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memory consolidation are mediated by distinct molecular processes; however, little is known regarding a potential mechanism driving these processes. The current study suggests that neocortical KOR activation mediates activation of these processes with learning. This study provides the first evidence for a time- and learning-dependent property of neocortical KOR in facilitating acquisition and consolidation of associative memories, while elucidating an unexplored neocortical learning mechanism.

Introduction

The opioid system has been extensively examined for its role in pain modulation; however, it has also been shown to play a prominent role in learning and memory. Nonspecific opioid receptor inhibition has been shown by multiple laboratories to impair learning for various behavioral tasks including shuttle avoidance, autoshaping, fear conditioning, Morris water maze, extinction paradigms and eyeblink conditioning (Izquierdo et al., 1980, Messing et al., 1989, McNally et al., 2004, Meilandt et al., 2004, Kim and Richardson, 2009). More precise studies exploring the specific opioid receptor mediating many of these learning effects have predominately focused on the mu-opioid receptor (MOR). These studies have extensively explored the role of MOR in acquisition of various learning paradigms such as Morris water maze (Jamot et al., 2003, Jang et al., 2003, Meilandt et al., 2004), radial arm maze (Jamot et al., 2003), and fear conditioning (Sanders et al., 2005). Likewise, our laboratory has demonstrated that blocking MOR systemically impairs acquisition for the forebrain dependent associative paradigm Whisker-Trace Eyeblink conditioning (WTEB) (Loh and Galvez, 2014).

In addition to a role for MOR in learning and memory, studies have recently demonstrated that the kappa-opioid receptor (KOR) also plays a critical role in acquisition of many behavioral tasks. For example, KOR stimulation via dynorphin prevents ischemic- (Itoh et

al., 1993c) or scopolamine-induced (Itoh et al., 1993b) deficits with spontaneous alternation. Likewise, prefrontal injections of KOR agonists impair alcohol reinstatement (Marchant et al., 2010), and withdrawal-induced conditioned place aversion (Kelsey et al., 2015). Furthermore, KOR knockout mice exhibit fewer errors on the radial arm maze, and decreased escape latency across several days of training in the Morris water maze (Jamot et al., 2003). KOR antagonists have also been shown to block acquisition of contextual fear conditioning in rats (Fanselow et al., 1991) and delay eyeblink conditioning in rabbits (Schindler et al., 1986, Schindler et al., 1987). These studies have suggested that KOR modulation can directly alter acquisition for various learning paradigms. In support of this hypothesis, our laboratory has also demonstrated that neocortical KOR modulation can alter acquisition for the forebrain dependent associative paradigm WTEB (Loh and Galvez, 2015).

Trace eyeblink conditioning is a well characterized and robust form of associative conditioning in which a neutral conditioning stimulus (CS), is paired with an unconditioned stimulus (US) that elicits an unconditioned response (UR) with a stimulus-free trace interval temporally separating the two. This paradigm is both dependent upon and stimulates learning induced changes in forebrain structures such as S1 and hippocampus (Solomon et al., 1986, Moyer et al., 1990, Kim et al., 1995, Weiss et al., 1999, Takehara et al., 2002, Galvez et al., 2007). Using whisker stimulation as the CS our laboratory has shown that learning results in S1 dendritic spine reorganization, suggesting that S1 is a site of storage for the trace association (Chau et al., 2014a). In exploring the role of KOR in these forebrain dependent neuronal mechanisms, we have further demonstrated that local infusions of the KOR specific antagonist, NorBNI hinders acquisition for the WTEB association (Loh and Galvez, 2015). These data suggest that KOR activation in S1 facilitates acquisition of the trace association.

Interestingly, KOR activation is known to exhibit a biphasic pattern with two conformational states that activate distinct molecular pathways (Bruchas and Chavkin, 2010). Upon initial KOR stimulation, the receptor activates Gα and Gβγ subunits that activate the intracellular kinases PI3K, PKCζ, ERK1/2, and JNK (Bruchas and Chavkin, 2010). A summary of this process is outlined in Figure 10. Interestingly, many of these kinases have also been implicated in acquisition of various learning paradigms. For example, PI3K in the hippocampus is required for the acquisition of conditioned place preference (Cui et al., 2010) and inhibition of PKC is capable of disrupting early memory formation in the mPFC (Evuarherhe et al., 2014). Additionally, ERK1/2 and its substrates are activated in the cerebellum immediately following eyeblink conditioning in rabbits (Zhen et al., 2001). These studies suggest that this initial KOR activation could facilitate kinase activation necessary for task acquisition.

With continued KOR stimulation, GRK3 will phosphorylate the KOR receptor and induce arrestin-dependent activation of the transcription factors pCREB and Zif268 (Bruchas and Chavkin, 2010). Similar to the kinases, these transcription factors can modulate learning; however, they have been shown to mediate consolidation rather than acquisition. For example, genetically or pharmacologically inhibiting CREB hinders consolidation of fear conditioning and water maze learning without altering acquisition or short term memories (Guzowski and McGaugh, 1997, Kida et al., 2002). Furthermore, Morris water maze studies have demonstrated that zif268 exhibits increased expression in the medial prefrontal cortex during periods of memory consolidation (Barry et al., 2016). These studies further suggest that this subsequent phase of KOR activation can drive transcription factor activation mediating memory consolidation. Following this final stage of KOR activation, it undergoes arrestin-dependent

internalization where it will either be degraded or recycled back into the membrane (Li et al., 1999).

Our initial study of neocortical KOR antagonism on forebrain dependent associative learning blocked both states of KOR activation, hindering our ability to determine the specific role for each state in acquisition and consolidation of the trace association. The current study used pharmacological inhibition for each state of KOR activation to determine their specific role in learning forebrain dependent associations.

Materials and Methods

Animals

Seventy-six 3-6 month old male C57BL/6 mice were bred in-house and housed in same-litter groups until surgery, where they were transferred to individual housing in standard (12”x12”x12”) laboratory cages. All mice were kept on a 12-hr light-dark schedule (lights on at 0700) in a temperature controlled room (~21° C), and provided *ad libitum* access to food and water. All procedures performed were reviewed and approved by the University of Illinois Animal Care and Use Committee and follow the National Institute of Health’s animal care guidelines.

Surgery

Surgeries were performed as previously described (Galvez et al., 2009). Mice were placed under ketamine (1mg/kg i.p.) and xylazine (6mg/kg i.p.) anesthesia. Once anesthetized, a headgear consisting of a plastic strip connector with two Teflon-coated stainless steel wires and one uncoated ground wire were secured to the skull via dental cement. Teflon-coated wires from the headgear were fed under the skin to the periorbital region of the eye, stripped to provide contact, and fastened to the skin. A ground wire was tightly secured to a screw in the skull. For

intra-S1 injections, a 26-gauge stainless steel guide cannula (4mm in length; PlasticsOne, Roanoke, VA) was inserted into S1 contralateral to the periorbital wire stimulators (-0.8mm AP, 3mm ML from bregma, and -0.5mm DV from the dorsal surface of the brain; (Franklin, 2008)). The guide cannula was secured to the headgear that was affixed to the skull with dental cement. Upon completion of the surgery, an obdurator was screwed into the guide cannula. All mice were given a minimum of seven days to recover from surgery before onset of training.

Behavioral Training

Mice were placed into standard laboratory cages (12”x12”x12”) different from their home cage in a sound attenuated chamber. All WTEB training took place between the hours of 0900 and 1400. The headgear described in the above section was connected to a tether that was then connected to a computer running a custom LabView program. The program delivered stimuli (both whisker and shock), as well as monitored eyelid closure. The tether allowed for freedom of mobility during all training procedures. One day prior to training, mice were habituated to the tether and chamber for 10 minutes. On training days, mice were conditioned as previously described (Galvez et al., 2009). A presentation of the CS (250ms whisker stimulation) was paired with a US (100ms periorbital shock, 0.1-0.5 mA square wave shock, 60Hz, 0.5ms pulses). The US shock intensity was tailored to each mouse to generate a detectable blink response with minimal voltage. This also allowed for a subsequent analysis of any drug effects on the shock intensity needed to induce a blink, as discussed in the results section below. The CS and US were separated by a 250ms stimulus-free trace interval (Figure 11). Mice were presented with the CS-US pairings 30 times per session (day) with an intertrial interval of 15-25 seconds. To monitor eyelid closure a camera on the tether provided a live video feed of the eye that was converted to a binary image in LabView in real time. Upon closure of the eyelid, the

size of the visible eye decreases indicating a blink (Figure 11). A CR was defined as a 4-standard deviation change in size of the eye binary image from baseline, occurring after CS onset and within 20ms prior to US onset (Figure 11). Baseline was defined as the average size of the eye binary image approximately 60ms prior to CS onset for each trial. These settings are consistent with that used in other laboratories conducting eyeblink analyses (Moyer et al., 1990, Tseng et al., 2004, Weiss and Disterhoft, 2011). Mice received 1 training session per day until behavioral criterion was achieved. Behavioral criterion was defined as exhibiting four CRs in five consecutive trials. Previous studies from our laboratory have demonstrated this behavioral criterion point to be consistent with acquisition of the trace association (Chau et al., 2013, Loh and Galvez, 2014, 2015). Upon achieving criterion, mice were allowed to complete the training session and then either sacrificed after one hour for KOR protein expression or returned to their home cage, where they remained for 30 days. After 30 days mice underwent re-testing, where they were again exposed to the same CS-US paired stimuli until behavioral criterion was again achieved. Re-exposure to CS-US paired stimuli is a standard procedure for assessing memory retention with WTEB conditioning (Galvez et al., 2011a, Schroeder et al., 2016). Although this paradigm can result in relearning of the association, behavioral performance of a well learned association would be apparent early in testing, minimizing any relearning. Furthermore, this paradigm allows for subsequent analyses of relearning the association due to initial drug induced altered acquisition/consolidation.

Conditioned Stimulus (whisker) stimulation control

A separate group of 9 male C57BL/6 mice aged 3 months underwent similar surgery as outline above. After one week of recovery, mice were injected with either PTX (n=3), GNTI (n=3), or saline (n=3). Twenty-four hours following injections, mice were placed in training

chambers with a custom tether for CS delivery to the whiskers contralateral to the injection hemisphere and eyelid closure monitoring. Mice were then exposed to stepwise increases in CS-only whisker stimulation. Whisker stimulation intensity was regulated by controlling the current delivered to the stimulator with a rheostat that varied from 0-100%. A camera focused on the rodent's eye ipsilateral to the injection hemisphere was used to determine when a blink occurred as described above. This procedure was performed to control for the effects of PTX or GNTI on whisker stimulation salience.

Examination of KOR-activated kinases on acquisition

A total of 18 male C57BL/6 mice, aged 3–6 months were used [PTX (2.0µg) n = 9, Saline n = 9]. Following habituation, half of the mice were randomly selected to receive S1 injections of PTX (2.0µg/animal, ListBio; Campbell CA) while the other half received saline. All injections were unilateral into S1 contralateral to whisker stimulation. PTX is a toxin that selectively catalyzes the ADP-ribosylation of G-proteins, preventing KOR-dependent kinase activation (Hsia et al., 1984). At the concentration used, PTX blocks KOR mediated behaviors such as morphine self-administration and behavioral sensitization (Self and Stein, 1993, Hummel and Unterwald, 2003). Additionally, Kanbara and colleagues (2014) have demonstrated that a single PTX injection can block morphine dependent behaviors for up to 7 days, suggesting that it has a prolonged duration of action on KOR activation (Kanbara et al., 2014). Interestingly, PTX does not impair arrestin-dependent KOR transcription factor activation (Li et al., 1999), demonstrating that it does not impair the second state of KOR activation (Figure 10). S1 PTX injections were conducted at a flow rate of 0.5 µl/min for 2 min for a total volume of 1µl (Hamilton 25µl gastight syringe, Fisher Scientific, Waltham, MA; Legato 101 pump; KD Scientific, Hollison MA). Upon completion of the infusion, the injector needle was left in place

for 1 minute to ensure diffusion of drug was complete. Approximately 24 hours after the injection, mice began WTEB training followed by retesting as outlined above. To date, there have been no studies demonstrating the effects of PTX lasting longer than 7 days, suggesting that mice were not under the influence of PTX during retesting; however, this cannot be completely ruled out in the current study.

Examination of KOR-activated arrestin-dependent transcription factors on consolidation

A total of 17, 3-6 month old male C57BL/6 mice (GNTI (20µg) n = 11, Saline n = 6) were trained on WTEB. Similar to experiment 1, following habituation, mice were randomly assigned to receive either GNTI (20ug/animal, Sigma Aldrich; G3416) or saline. All injections were again conducted unilaterally into S1 contralateral to whisker stimulation. The volume and flow rate of the injections were conducted as outlined in the PTX study. GNTI effectively blocks the latter portion of KOR activation via inhibiting arrestin-dependent mechanisms, without effecting initial activation of the receptor (Rives et al., 2012). Approximately 24 hours after the injection, mice began WTEB training followed by retesting as outlined above. Note, GNTI provides a strong KOR specific antagonism that in vivo has been shown to last for approximately 28 days, after which the overall KOR antagonism drops nearly 100-fold (Munro et al., 2012), suggesting that it would effectively antagonize KOR during training but not retention testing. These data further suggest that any behavioral differences during retention testing are not due to continued GNTI-mediated KOR antagonism.

Examination of KOR-protein expression following learning

For examination of learning-induced KOR expression in S1 of conditioned mice (COND), two additional control groups were used. Unpaired mice (Unpaired) were provided 30 unpaired CS and US presentations per session (day) with an intertrial interval of 15-25 seconds.

This group has been referred to as “pseudo conditioned” in prior studies and served as a stimulation control. In addition, naïve cage control mice (CC) were used to determine if stimulation alone in the absence of learning (Unpaired) altered KOR expression. One-hour following reaching behavioral criterion, COND mice (n=8) were given an overdose of sodium pentobarbital and transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Unpaired mice (n = 12) were yoked to a COND mouse to determine when to discontinue training. This procedure ensured that the unpaired mouse received similar amounts of stimulation, without acquiring the association. CC mice (n = 12) were also collected at this time. The brains were then collected and placed into 4% paraformaldehyde overnight at 4° C and transferred into 30% sucrose until sectioned. S1 was then coronally sectioned at 30 μm and stored in cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1M PBS).

KOR protein was then visualized in the sections using a standard immunohistochemistry protocol. Briefly, sections were washed several times in PBS, treated with 0.6% H_2O_2, blocked in PBS-X (2% normal goat serum and 0.5% triton-x in PBS) for 1 h at room temperature and then incubated overnight at 4° C in anti-KOR antibody (Oprk1: 1:500 MBL International Corporation, Woburn, MA, USA). Three sections per animal spanning S1 were then washed in PBS-X, incubated for 2 h in secondary antibody (Goat-Anti-Rabbit 1:100) at room temperature, washed in PBS-X and treated with avidin-biotin amplification (Vectastain) for 1 h. Following additional PBS washes, the KOR expression was visualized with diaminobenzidine (0.5 mg/ml DAB, 6.95 mg/ml nickel ammonium sulfate, 0.033 µl 30% hydrogen peroxide) detection.

For KOR protein analysis, a digital image of S1 was captured with the Zeiss AxioCam ICc 1 camera at 10x magnification. KOR positive puncta greater than 30μm^2 were then counted

(counting frame = 635µm x 475µm) using the Particle Analyze feature on ImageJ (Version 1.45s, NIH). An individual puncta was defined as an areas containing DAB precipitate and thus should not be thought of as a specific cell count, but rather KOR density. Images taken at 10x magnification contained all of S1. Note all data collection and treatment of images were conducted blind to the experimental group.

Histology

Upon completion of the experiment, mice were given a lethal dose of 50mg/ml pentobarbital, transcardially perfused with 0.1M PBS (Ph=7.4) followed by 4% paraformaldehyde and the brains removed. The brain was then post-fixed for 24 hours in 4% paraformaldehyde, stored in 30% sucrose solution and sectioned in 30 µm sections. For mice that received intracranial injections, sections spanning the injection site were then nissl stained with Cresyl Violet to verify cannula placement in S1.

Statistics

A one-way analysis of variance (ANOVA) was used to analyze days to criterion. The analysis of days to criterion will determine if the drug has an effect on the rate of acquisition. To determine if differences in the days to criterion are due to a gradual acquisition over a longer period of time or represent a shift in acquisition to a delayed time-point, behavioral performance was normalized to day of criterion. This procedure controls for individual animal learning variability while allowing for examination of drug effects on acquisition curves. Specifically, behavioral performance was normalized to the day each mouse reaches behavioral criterion (C, 4 CRs out of 5 CS-US pairings) and the four days immediately preceding C (C1, C2, C3, C4). This procedure controls for individual animal learning variability while allowing for examination of drug effects on acquisition curves. Additionally, this normalization better controls for individual

differences in animal learning. These learning curves and non-normalized behavioral performance across successive days were examined with a two-way ANOVA with repeated measures and degrees of freedom adjusted via Kenward-Rogers. As there were no differences observed on C4 with all mice performing at 0% conditioned responses, data from C4 was removed from analysis, and all subsequent analyses contain only day of criterion (C), and the three days immediately preceding C. To determine differences in KOR protein expression an ANOVA with a Fisher's LSD and Bonferroni correction for multiple comparisons was used. All analyses were conducted with SAS 9.4.

Results

Examination of KOR-activated kinases on acquisition

Histological examination of cannula location demonstrated that all mice had received the injection in S1 (Figure 12). Upon examination of the behavioral data, an overall ANOVA demonstrated a significant effect of drug condition on the number of days to reach behavioral criterion ($F_{(1, 16)} = 9.18$, $p = 0.008$). PTX treated mice took an average of 5.33 (± 0.57) days to reach behavioral criterion, while saline treated mice took an average of 2.89 (± 0.57) days to reach behavioral criterion, demonstrating that PTX impaired acquisition for the trace association (Figure 13A). After normalizing the behavioral data to the day of criterion for each animal, a repeated measures ANOVA with Kenward-Rogers degrees of freedom adjustment demonstrated that all groups acquired the task across criterion days (C3, C2, C1, C) ($F_{(3,36.6)} = 49.67$, $p < 0.0001$); however, there were no significant differences between the groups or a group by day interaction, suggesting that PTX did not alter the rate of acquisition. To determine the effects of PTX on consolidation, mice were then retested following 30 days of no training. When retested, an overall ANOVA demonstrated no significant difference in the number of days needed to again

reach behavioral criterion between drug groups. PTX treated mice took an average of 1.17 (± 0.10) days to reach behavioral criterion, while saline treated mice took an average of 1.00 (± 0.08) days to reach behavioral criterion, suggesting that PTX did not alter consolidation for the trace association. Interestingly, examination of the percent conditioned response on the first retention day (R1), demonstrated a significant difference between saline and PTX treated mice (Figure 13B). This was primarily due to an increase in performance in saline treated mice, as the PTX %CR on R1 was not significantly different from %CR on acquisition day C.

To examine possible PTX induced KOR modulation of sensory systems unrelated to learning, threshold shock intensities and whisker stimulation were examined. Threshold shock intensities for animals that received PTX or saline did not significantly differ. PTX treated mice needed an average of 0.13 (± 0.02) mV to exhibit a blink, while saline treated mice needed an average of 0.11 (± 0.02) mV to exhibit a blink, suggesting that PTX induced KOR modulation in S1 does not significantly alter the ability to detect the shock relative to saline injected controls (Figure 14). Similar to US detection, there was no significant difference in any group's ability to detect the conditioned stimulus whisker stimulation. PTX treated mice required an average of 88.33% (± 2.72) stimulation before displaying a blink, while saline treated mice required an average of 90.00% (± 2.72) stimulation before displaying a blink, suggesting that PTX induced KOR modulation in S1 does not significantly alter the ability to detect the whisker stimulation relative to saline injected controls (Figure 14). These findings are consistent with previous studies from our laboratory demonstrating no significant effects in the shock intensity needed to induce a blink, whisker detection, or blink properties following either a general opioid or KOR specific antagonist (Loh and Galvez, 2014, 2015).

Examination of KOR-activated arrestin-dependent transcription factors on consolidation

Histological examination of cannula location demonstrated that all mice had received the injection in S1 (Figure 12). Upon examination of the number of days needed to reach behavioral criterion no significant differences between GNTI and saline treated mice were observed. GNTI treated mice took an average of 4.72 (± 0.63) days to reach behavioral criterion, while saline treated mice took an average of 3.50 (± 0.85) days to reach behavioral criterion, suggesting that GNTI does not hinder acquisition for the trace association (Figure 15A). After normalizing the behavioral data to the day of criterion for each animal, a repeated measures ANOVA with Kenward-Rogers degrees of freedom adjustment demonstrated that all groups acquired the task across criterion days (C3, C2, C1, C) ($F_{(3, 67.9)} = 41.12$, $p < 0.0001$) and that there was no significant group effect or group by day interaction (Figure 15B). To determine the effects of GNTI on consolidation of the WTEB association, mice were then retested 30 days after reaching behavioral criterion. When mice were retested an overall ANOVA demonstrated a significant difference in the number of days required to reach behavioral criterion between drug groups $F_{(1,15)} = 66.04$, $p < 0.001$. GNTI treated mice took an average of 3.64 (± 0.18) days to reach behavioral criterion, while saline treated mice took an average of 1.16 (± 0.24) days to reach behavioral criterion (Figure 15A). These data suggest that while GNTI did not hinder acquisition, it impaired consolidation of the trace association. Upon completion of retention testing, the percent CR on that day for each mouse was compared between saline and GNTI treated mice. There was no significant difference between groups, demonstrating that GNTI treated mice eventually reached the same level of behavioral performance as observed in saline treated mice. Additionally, normalizing the GNTI retention testing to the day of behavioral criterion demonstrated no significant difference when compared to GNTI or saline across

acquisition criterion days (C3, C2, C1, C). These findings suggest that there was no savings in GNTI treated mice from the initial acquisition, thus further suggesting that unlike saline treated, GNTI treated mice exhibited impaired consolidation and had to relearn the trace association.

To examine possible GNTI induced KOR modulation of sensory systems unrelated to learning, threshold shock intensities and whisker stimulation were examined. Threshold shock intensities for animals that received GNTI did not significantly differ from saline controls. GNTI treated mice needed an average of 0.21 (± 0.009) mV to exhibit a blink, while saline treated mice needed an average of 0.21 (± 0.01) mV to exhibit a blink, suggesting that GNTI induced KOR modulation in S1 does not significantly alter the ability to detect the shock relative to saline injected controls (Figure 14). It should be noted that there was a significant difference between experiments (PTX/PTX-Saline vs. GNTI/GNTI-Saline); however, there was no interaction between drug and experiment, indicating that this was not due to the drug treatment but rather an effect of the individual run. Furthermore, as discussed above PTX and GNTI at the concentrations administered into S1 were not found to alter the shock intensity needed to induce a blink or any measured blink properties. In addition, there was no significant difference in any group's ability to detect the conditioned stimulus whisker stimulation. GNTI treated mice required an average of 91.67% (± 2.72) stimulation before displaying a blink, while saline treated mice required an average of 90.00% (± 2.72) stimulation before displaying a blink, suggesting that GNTI induced KOR modulation in S1 does not significantly alter the ability to detect the whisker stimulation relative to saline injected controls (Figure 14). These findings are consistent with prior studies from our laboratory utilizing either a general opioid or KOR specific antagonist (Loh and Galvez, 2014, 2015).

Examination of KOR-protein expression following learning

Analysis of acquisition for the trace association with a two-way ANOVA demonstrated a significant difference between groups ($F_{(1,44)} = 10.90$, $p < 0.05$), acquisition across days ($F_{(3,44)} = 14.56$, $p < 0.05$), and interaction between groups and acquisition curves ($F_{(3,44)} = 8.66$, $p < 0.05$; Figure 16A). These findings suggest that COND trained mice acquired the association to a significantly higher degree than Unpaired mice. Post-hoc analyses using the Tukey criterion for significance indicated that on criterion day, COND mice performed significantly better with an average %CR of 46.25 (± 3.34) compared to Unpaired mice who had an average %CR of 10.91 (±2.88). Analysis of KOR expression in S1 yielded a significant overall one-way ANOVA with an effect of group ($F_{(2,15)} = 9.22$, $p < 0.05$; Figure 16B). Post-hoc analyses using Fisher's LSD criterion for significance demonstrated that COND mice had significantly fewer KOR+ puncta than Unpaired or cage controls (CC). COND mice had an average of 0.00224/μm^2 (± 0.000114) KOR+ puncta while Unpaired mice had an average of 0.00274/μm^2 (± 0.000316) puncta and cage controls had 0.00302/μm^2 (± 0.000388) puncta. Consistent with the known pathway for KOR dependent activation (Figure 10), these findings suggest that with WTEB conditioning KOR in S1 is activated and subsequently downregulated.

Discussion

In exploring the role of the Opioid system in learning and memory many studies have focused primarily on the mu-subtype; however, recent findings both from our laboratory and others have demonstrated that the kappa-subtype also plays a prominent role. The current study found that the distinct phases of KOR activation (early g-protein-mediated kinase activation vs. late arrestin-dependent phosphorylation followed by transcription factor activation) have differential effects on the learning process. Specifically, disruptions in this early phase vs. the

later phase hinders acquisition and consolidation respectively. Additionally, this study demonstrated that upon learning, there is a significant decrease in the amount of KOR protein in S1 when compared to unpaired mice and cage controls, suggesting KOR activation with learning.

In the current study, the initial phase of KOR activation was antagonized in S1 contralateral to whisker stimulation with PTX and selectively attenuated acquisition for the associative paradigm, WTEB. Although the specificity of PTX is not exclusively to KOR, the observed results are consistent with our prior findings demonstrating that KOR specific antagonism into S1 impairs acquisition for WTEB (Loh and Galvez, 2015). Furthermore, the actions of PTX on KOR have been well documented (Bruchas and Chavkin, 2010) with it inhibiting KOR-dependent kinase activation, while not affecting downstream arrestin-dependent mechanisms and transcription factor recruitment (Li et al., 1999). This finding suggests that initial KOR-dependent kinase activation is an important mediator of early acquisition of forebrain-dependent learning tasks such as WTEB. Interestingly, similar to our prior findings using the general KOR antagonist NorBNI (Loh and Galvez, 2015), PTX impaired but did not prevent, acquisition for the WTEB association.

The finding that blocking KOR-mediated activation is capable of delaying acquisition of the association is also consistent with other studies. KOR antagonism has been shown to delay acquisition for a conditioned place aversion task (Tejeda et al., 2013, Kelsey et al., 2015) and impair the memory protective effects of KOR activation (Itoh et al., 1993a, b, c). We have also shown that KOR antagonism via NorBNI either systemically or locally hinders WTEB acquisition (Loh and Galvez, 2015). These studies collectively suggest that neocortical KOR activation facilitates learning. Interestingly some studies have found that KOR activation impairs

rather than facilitates learning. For example, endogenously activated KOR impairs novel object recognition, and the effects are ameliorated with deletion of the gene encoding the KOR ligand, dynorphin (Carey et al., 2009). Furthermore, genetic removal of KOR facilitates learning on the Morris water maze and radial arm maze tasks (Jamot et al., 2003). These studies collectively suggest that the role of KOR in learning is not simple, and that it may be working through an inverted U function, with significant differences depending on factors that can induce KOR endogenous ligand release, such as stress. Subsequent studies will need to pay close attention to these factors in their interpretations of the role of KOR in learning.

Our finding that PTX hinders acquisition for the WTEB association is novel; however, the KOR-dependent downstream mechanisms blocked by PTX (Figure 10) have an extensive history of being involved in learning and memory. For instance, PI3K in the hippocampus is required for acquisition of conditioned place preference (Cui et al., 2010), while PKC inhibition shows detriments to early mPFC dependent memory formation (Evuarherhe et al., 2014). These kinases are directly activated via the downstream activity of KOR, and they have a clear role in the acquisition of learned associations. For a detailed account of KOR-dependent mechanisms see Bruchas and Chavkin (2010) (Bruchas and Chavkin, 2010).

Once the association was acquired, PTX-treated mice exhibited normal long-term consolidation of the association. PTX-treated mice did not significantly differ in the number of days required to once again reach behavioral criterion in 30-day retention tests from saline-treated mice, suggesting that PTX while impairing acquisition, did not hinder consolidation of the association. Interestingly, on day 1 of retention testing (R1, Figure 13B) saline-treated mice exhibited significantly better performance (%CR) than PTX treated; however, this was due to a significant increase in the saline-treated mice from the last day of training. Although the specific

mechanism(s) that may be mediating this increase in behavioral performance following a delay is not known, Hoffman and coworkers (2002) have proposed offline reactivation as one possibility that would be consistent with our data (Hoffman and McNaughton, 2002). This process of reactivation of the memory trace is believed to play a prominent role in learning mildly aversive stimuli such as WTEB (Valdes et al., 2015). Given that this mechanism should work through similar learning mechanisms as employed during initial presentation of the training stimuli, one would expect it to also require kinase activation and thus be sensitive to PTX treatment. As an interesting note, we did not see this same increase in behavioral performance in our examination of the arrestin dependent factors (Figure 15). Additional studies will be required to further investigate these findings.

In addition to demonstrating that the initial phase of KOR activation facilitates task acquisition, the current study demonstrated that selectively blocking the later phase of KOR activation in S1 with GNTI did not significantly affect initial acquisition, but rather impaired consolidation of the WTEB association, as apparent from the 30-day retention testing. GNTI has a very high affinity for KOR, but only antagonizes arrestin-dependent KOR transcription factor recruitment (Bruchas and Chavkin, 2010). This specificity allows for regular activation of the KOR kinase cascade without the downstream activity of the transcription factors or arrestin-dependent internalization (Figure 10). We have demonstrated that GNTI in S1 blocked consolidation of the WTEB association, without effecting acquisition. These findings suggest that acquisition for the WTEB association is not dependent upon the later phase of KOR activation. These findings further suggest that this later phase of KOR activation, resulting in transcription factor activation (CREB and zif268) and receptor internalization, is critical for neocortical long term memory consolidation.

The current findings, that blocking activation of the transcription factors CREB and zif268 impair consolidation while not altering initial acquisition, are consistent with many learning studies. Genetic knockout of CREB hinders memory consolidation, while having no effect on acquisition in both water maze and fear conditioning (Bourtchuladze et al., 1994, Guzowski and McGaugh, 1997, Kida et al., 2002). Similarly, pharmacologically blocking upstream regulators of CREB impairs consolidation, while having no effect on acquisition of fear conditioning in mice (Bailey et al., 1999). Furthermore, mPFC zif268 expression has been shown to increase with time following a learning event, suggesting a role in memory consolidation (Barry et al., 2016). These findings collectively demonstrate that the downstream factors of late phase KOR activity (phosphorylation, arrestin recruitment, and transcription factor activation) are important for the formation of longer lasting memories. Utilizing the actions of GNTI, we have shown that inhibiting these processes leads to deficits in consolidation of WTEB.

Interestingly, while PTX and GNTI had different effects on the acquisition of the association, when standardized to day of criterion, they both exhibited similar rates of acquisition that did not differ from saline-treated controls. We previously observed similar findings when examining the effects of blocking both phases of KOR activation with NorBNI on WTEB acquisition (Loh and Galvez, 2015). We should also note that it was previously determined in that study that blocking both phases of KOR did not alter blink properties, whisker detection, or periorbital shock sensitivity; thus suggesting that these effects are not due to stimuli detection. A finding further supported by the analyses in the current study (Figure 14).

The final stage in arrestin-dependent KOR activation results in receptor internalization and downregulation. In support of this mechanism, our analysis of KOR protein expression following learning demonstrated a significant decrease in the amount of KOR protein in S1

relative to unpaired and cage-controls. These findings add significant support to the phasic properties and learning dependent neocortical activation of KOR. The biphasic activation pattern of KOR is an area of active investigation; however most studies have been conducted in vitro following agonist treatment. One such study has demonstrated that the initial kinase activation takes place within 10 minutes of treatment (Belcheva et al., 2005). Similarly, another study investigating the second phase of KOR activation has demonstrated that late phase ERK activation occurs approximately one hour after agonist treatment (Gesty-Palmer et al., 2006). Other studies have suggested that KOR is internalized approximately 12 hours following activation (Bhargava et al., 1989). Interestingly, these mechanisms (KOR dependent kinase activation, transcription factor activation, and receptor internalization) would only require KOR initial activation during learning. Once KOR is activated, these down-stream mechanisms would be engaged and based upon in vitro studies, become independent of continued KOR stimulation (Belcheva et al., 1998, Xu et al., 2007). Although it may initially seem counter intuitive to decrease expression of a receptor mediating learning, such a mechanism could facilitate the fine tuning of stimuli specific synaptic connections. Many studies have shown KOR to be localized to and able to modulate synapse specific processes (Reyes et al., 2009, Pennock and Hentges, 2011). A time-dependent reduction in synapse specific KOR expression would ensure that extraneous learning events did not hinder consolidation of the initial event. Decreasing KOR expression would allow for the strengthening of task specific synaptic connections consolidating the memory, while other synaptic connections and neuronal pathways are mediating different learning events. Further work will be needed to better decipher a functional role for this neocortical downregulation of KOR and its potential role in facilitating learning and memory consolidation.

Conclusion

This report 1) provides insight into an underlying mechanism mediating neocortical dependent task acquisition and memory consolidation, and 2) warns future researchers of the potential memory impairing effects of KOR antagonists. This is the first report of a behavioral task showing distinct modulatory effects of the biphasic activation of KOR. Additionally, this report outlines a time- and learning-dependent mechanism for KOR in facilitating both acquisition and consolidation of neocortical associative memories. The double dissociation outlined in this study adds interesting and compelling evidence for the role of KOR in neocortical dependent learning tasks, and suggests that different types of KOR modulators, that is, those that selectively inhibit or facilitate either phase of KOR activation, can have different effects task acquisition and memory consolidation.

Figures

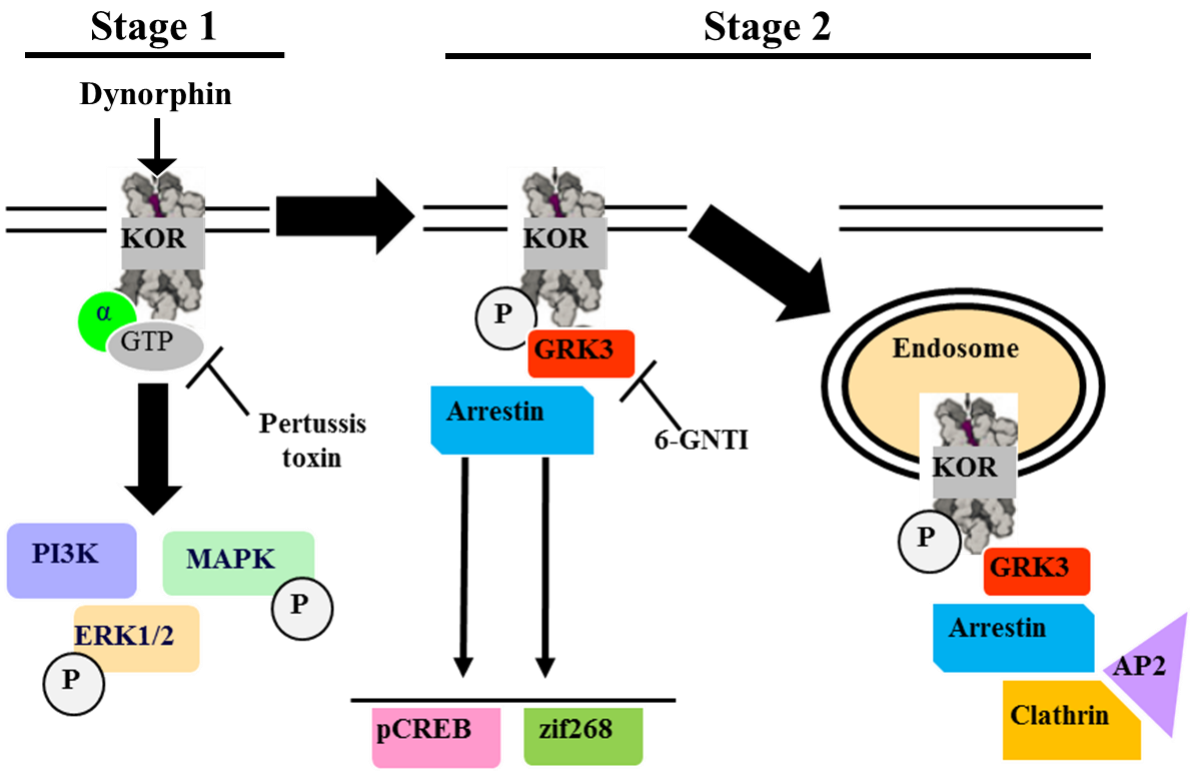

Figure 10: Summary diagram adapted from (Bruchas and Chavkin, 2010) outlining the different stages of KOR activation. Stage 1 ligand-signaled activation utilizes pertussis toxin sensitive G-proteins to activate intracellular kinases such as P13K, MAPK, and ERK1/2. Stage 2 utilizes 6-GNTI-sensitive GRK3 to activate arrestin and downstream transcription factors pCREB and Zif268. At the end of Stage 2 KOR expression is downregulated via arrestin-dependent internalization.

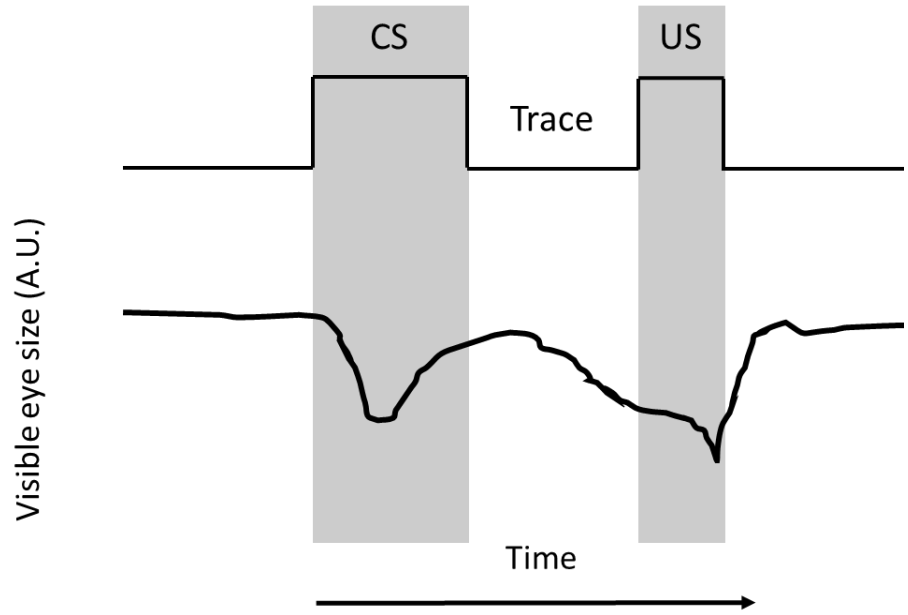

Figure 11: Schematic of Whisker-Trace Eyeblink (WTEB) paradigm. In WTEB a conditioned stimulus (CS; whisker stimulation), is paired with an unconditioned stimulus (US; periorbital shock) separated by a stimulus free interval (Trace). Upper line shows square wave computer delivered stimuli. Bottom line shows relative visible eye size in arbitrary units (A.U.). A downward deflection of the line represents a decrease in the size of the visible eye due to closure of the eyelid, i.e. a blink. Note, the blink (closing of the eye) during the pre-US interval illustrating a conditioned response (CR).

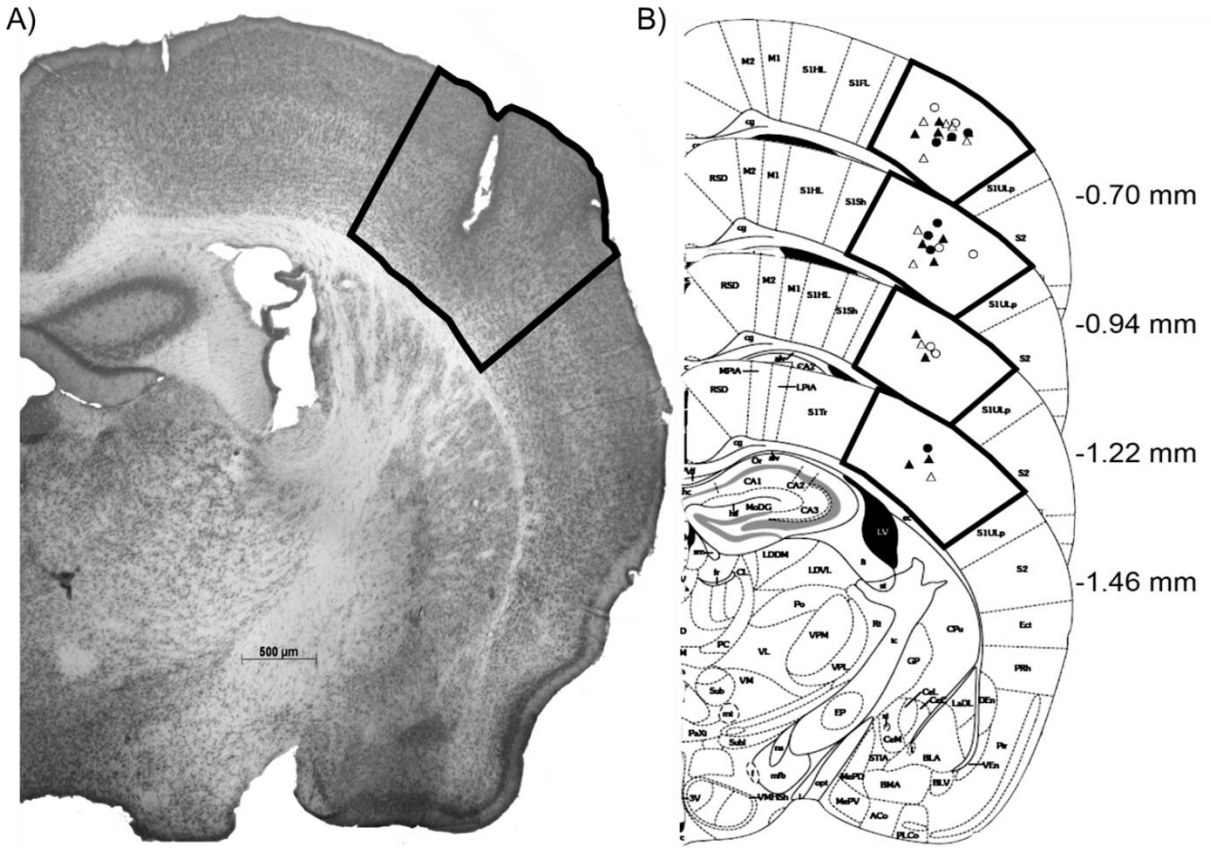

Figure 12: Diagram showing injection location for all mice across both intracranial studies. A) Representative nissl stained section illustrating the injection cannula track and location in primary somatosensory cortex (S1). B) Individual atlas images illustrating each injection location for both injection studies. Numbers to the right are the coordinates from bregma for each image. Note, all injections were within S1. For clarity, S1 has been outlined in both the atlas and nissl stained images. Open circle: PTX-saline. Closed circle: PTX. Open triangle: GNTI-saline. Closed triangle: GNTI.

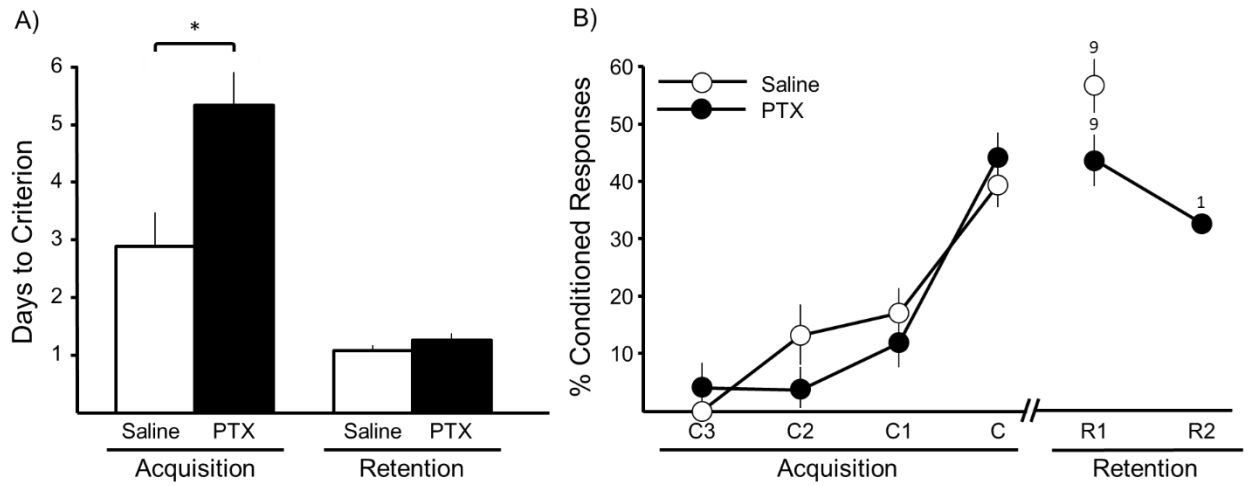

Figure 13: Pertussis Toxin (PTX) delays acquisition, but not retention of WTEB. A) Mice who received PTX into S1 took significantly longer to achieve behavioral criterion than those that received saline. Behavioral criterion was defined as exhibiting four conditioned responses (CRs) in five consecutive trials. When tested 30 days later (Retention), both PTX and saline groups reached behavioral criterion in a similar number of days. B) When learning curves were standardized to day of criterion (C = day of criterion; C1 = day before criterion; C2 = two-days before criterion; C3 = three-days before criterion), there was no significant difference between PTX and saline treated mice in their percent conditioned response rate of acquisition. When tested 30 days later (Retention), both PTX and saline groups retained the association. Training for each mouse was discontinued once it reached behavioral criterion during retention testing. All but one PTX mouse reached behavioral criterion on retention day 1 (R1). The number of mice at each retention day is delineated above the retention data points. Asterisk indicates $p < 0.05$.

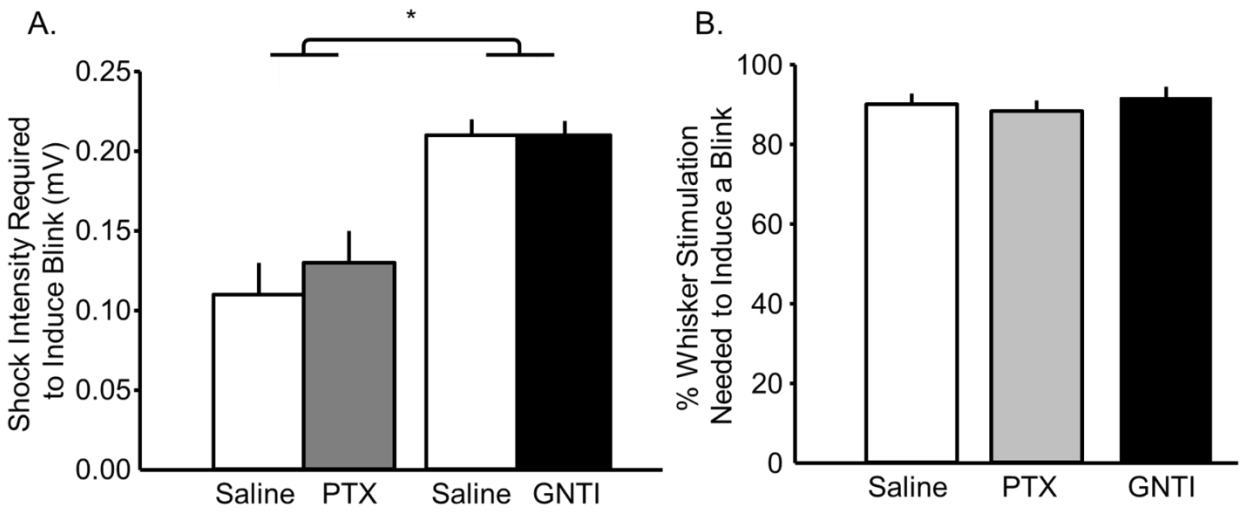

Figure 14: Neither PTX nor GNTI significantly alter detection of whisker or shock stimulation. A) Mean shock stimulation required for mice to elicit blink response. There was no significant difference in the shock threshold required to induce a blink within each study; however, the shock intensity was significant across experiments, independent of drug condition. There was no significant interaction between experiment and drug condition, demonstrating that this effect was not due to PTX or GNTI. B) Mean percent whisker stimulation required to elicit blink response from each group. There was no significant difference in the ability of mice to detect whisker stimulation as a result of any drug condition. Asterisk indicates $p < 0.05$.

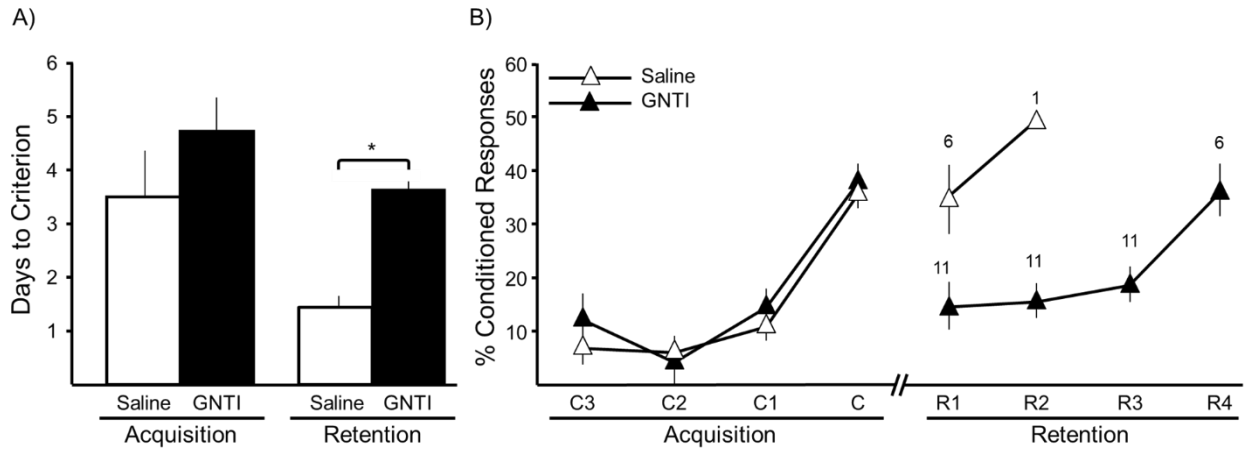

Figure 15: Guanidinonaltrindole (GNTI) injections into primary somatosensory cortex (S1) inhibit retention, but not acquisition of WTEB. A) Mice who received GNTI into S1 did not significantly differ in the number of days needed to reach behavioral criterion during acquisition of WTEB from saline controls. Behavioral criterion was defined as exhibiting four conditioned responses (CRs) in five consecutive trials. When tested 30 days later (Retention), GNTI treated mice took significantly longer to achieve behavioral criterion than saline controls. B) When learning curves were standardized to day of criterion (C = day of criterion; C1 = day before criterion; C2 = two days before criterion; C3 = three days before criterion), there was no significant difference in the rate of acquisition between GNTI and saline treated mice. Interestingly, during retention testing, GNTI treated mice exhibited similar rates to what was observed during initial acquisition (C3, C2, C1 and C) while saline treated mice all retained the association and reached behavioral criterion on retention day 1 and 2 (R1 & R2). Training for each mouse was discontinued once it reached behavioral criterion during retention testing. All but one saline mouse reached behavioral criterion on retention day 1 (R1). The number of mice at each retention day is delineated above the retention data points. Asterisk indicates $p < 0.05$.

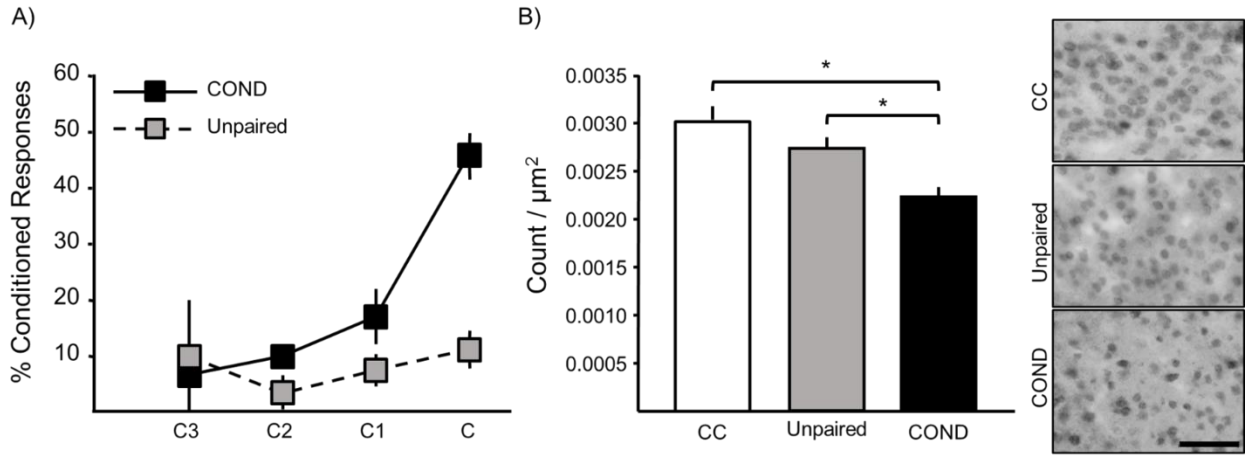

Figure 16: WTEB conditioning reduces neocortical KOR expression in primary somatosensory cortex (S1), consistent with arrestin-dependent internalization of KOR. A) Mean percent conditioned response (CR) per session until trace-paired conditioned (COND) mice reached criterion (C = day of criterion; C1 = day before criterion; C2 = two-days before criterion; C3 = three-days before criterion). Trace-unpaired mice (Unpaired) were yoked to COND mice to determine their criterion days. All COND mice exhibited a significant increase in percent CRs across criterion days and compared to Unpaired mice. B) KOR protein expression in somatosensory cortex is decreased following acquisition of the WTEB association. COND mice had significantly less KOR expression relative to Unpaired controls, and cage control (CC). Right: Representative images showing KOR puncta staining in CC, Unpaired, and COND mice. Scale bar = 50µm. Asterisks indicates $p < 0.05$.

Chapter 5 - Investigating an Upstream Component of Kappa Opioid Receptor-Dependent Learning in S1; A Fluorescent Analysis of Prodynorphin and Somatostatin

Abstract

There are several lines of evidence that indicate a prominent role for the opioid peptide system in the acquisition and consolidation of learned associations. Specifically, kappa opioid receptor (KOR) modulation has been demonstrated to alter various behavioral learning tasks such as inhibitory avoidance, spontaneous alternation, spatial maze, and conditioned place aversion. Likewise, our laboratory has demonstrated there to be a substantial role for KOR in both the acquisition and consolidation of whisker trace eyeblink conditioning (WTEB). WTEB is an associative conditioning paradigm in which a neutral conditioned stimulus (CS; Whisker stimulation) is paired following a short stimulus free trace interval with a salient unconditioned stimulus that elicits a blink response (US; Eye shock). Work from our laboratory has shown that WTEB conditioning is dependent upon and induces plasticity in the primary somatosensory cortex (S1), a likely site for memory storage. Our subsequent studies have shown that WTEB acquisition or consolidation are impaired when the initial or later phase of KOR activation in S1 is respectively blocked. Interestingly, this mechanism by which KOR is activated in S1 during learning has not been explored. Dynorphin (DYN), KOR's endogenous ligand, is synthesized in axon terminals from the precursor prodynorphin (PD) that is synthesized from preprodynorphin (PPD). In S1, most PPD is found in inhibitory GABAergic somatostatin interneurons, suggesting that these somatostatin-containing GABAergic interneurons (SOM) are upstream regulators of learning induced KOR activation. To determine their potential role in learning induced KOR activation, the current study used immunofluorescence in conjunction with WTEB to determine the time-dependent learning induced PD/SOM expression pattern.

Introduction

Opioid peptides have a long history of involvement in pain, reward, and learning and memory. Previous reports from our laboratory and others have demonstrated that the general opioid antagonist naloxone, impairs learning of many associative tasks such as delay conditioning, operant lever-pressing behavior, fear conditioning, and trace eyeblink conditioning (Izquierdo et al., 1980, Hernandez and Powell, 1983, Messing et al., 1989, Kim and Richardson, 2009, Loh and Galvez, 2014). In exploring the role for specific opioid receptors in learning and memory, our laboratory has shown that antagonizing the kappa-opioid receptor (KOR) impairs acquisition for the associative paradigm whisker-trace eyeblink (WTEB) conditioning (Loh and Galvez, 2015).

In WTEB conditioning a neutral conditioned stimulus (CS; Whisker stimulation) is paired following a stimulus free trace interval with a salient unconditioned stimulus that elicits an eye-blink (US; Eye shock). This paradigm has been shown to be dependent on, and induce plasticity in primary somatosensory cortex (S1), a likely site for memory storage (Galvez et al., 2006, Galvez et al., 2007, Chau et al., 2013, Chau et al., 2014a). Our studies exploring the role of KOR with learning have demonstrated that either systemic or direct S1 KOR inhibition impairs WTEB acquisition (Loh and Galvez, 2015). Consistent with our findings, many studies have shown that KOR modulation can alter learning on various tasks such as inhibitory avoidance, spontaneous alternation, water- and radial arm mazes, and conditioned place aversion (Ilyutchenok and Dubrovina, 1995, Ukai et al., 1995, Jamot et al., 2003, Tejeda et al., 2013, Kelsey et al., 2015). These studies have strongly suggested that KOR plays a prominent role in mediating various types of learning.

KOR's endogenous ligand and the most likely molecular trigger for these effects on learning is dynorphin. Dynorphin is synthesized from the precursor peptide prodynorphin (PD), which is synthesized in vesicles from the precursor preprodynorphin (PPD) (Day et al., 1998). Similar to KOR, Dynorphin and its upstream precursors have been implicated in various forms of learning. For instance, PD knockout mice exhibit enhanced levels of freezing in a contextual fear conditioning task (Bilkei-Gorzo et al., 2012). Likewise, dynorphin knockout mice display reduced age-related deficits in water maze learning (Nguyen et al., 2005), while dynorphin injected directly into the hippocampus impairs water maze performance (Sandin et al., 1998). Although the specific role of dynorphin and its precursors in learning appears to vary depending upon the tasks, these studies collectively suggest that they play a prominent role in the acquisition and consolidation of learning tasks.

Upon exploring the specific cell type expressing these peptides, it has been shown that the dynorphin precursor ligand PPD is primarily found in Somatostatin-containing GABAergic interneurons (SOM) within S1 (Sohn et al., 2014). These findings suggest that SOM interneurons are a likely upstream cell of KOR activation in S1 with WTEB. SOMs are a major subclass of interneurons in the central nervous system that serve a variety of roles in various species. SOM cells represent a significant proportion of all inhibitory interneurons in the neocortex (Lee et al., 2010). Additionally, this population of cells is localized in areas important for learning and memory, such as the neocortex and hippocampus (Fino and Yuste, 2011), two brain regions critically involved in WTEB acquisition (Solomon et al., 1986, Power et al., 1997, Galvez et al., 2007). In S1, tonically-active SOM cells serve to inhibit neuronal activity, as optogenetic silencing SOM cells causes an increase in firing of pyramidal cells in the area. SOM cells in S1 receive input from vasoactive intestinal peptide (VIP)-containing inhibitory interneurons.

Interestingly, during active whisking these cells cause a decrease in SOM activity (Lee et al., 2013). This process does not seem to be dependent upon motor input, as passive whisking will also instigate the same mechanism via thalamic relays (Gentet et al., 2012).

Similar to that observed with KOR modulation, studies have strongly suggested a role for SOM cells in learning. Silencing SOM cells in the hippocampus impairs acquisition of contextual-based fear learning (Lovett-Barron et al., 2014). SOM cells are also disinhibited by VIP-containing inhibitory neurons during auditory discrimination (Pi et al., 2013). This VIP disinhibition is similar to what is seen in S1 during active (Lee et al., 2013), or passive whisking (Gentet et al., 2012). These studies suggest that neocortical SOM cells regulate learning processes. Furthermore, these studies along with those mentioned above collectively suggest that neocortical SOM cells are regulating learning through KOR modulation. However, the specific role for neocortical SOM regulation of KOR activity with associative learning has never been explored. To explore this molecular pathway and its potential role with associative learning, the current study set out to characterize the expression profile of PD (Dynorphin's precursor) in S1 SOM interneurons during and immediately following WTEB acquisition.

Methods

Animals

Three to six month old male C57BL/6 mice were bred in-house and housed in same litter groups until surgery. After surgery they were transferred to individual housing in standard (12"x12"x12") laboratory cages. All mice were kept on a 12-hour light-dark schedule (lights on at 0700) in a temperature controlled room (~21º C) and provided *ad libitum* access to food and water. All procedures performed were reviewed and approved by the University of Illinois Animal Care and Use Committee.

Surgery

Surgeries were performed as previously described (Galvez et al., 2009). Mice were placed under ketamine (1mg/kg i.p.) and xylazine (6mg/kg i.p.) anesthesia. Once anesthetized, a headgear consisting of a plastic strip connector with two Teflon-coated stainless steel wires and one uncoated ground wire were secured to the skull via dental cement. Teflon-coated wires from the headgear were fed under the skin to the periorbital region of the eye, stripped to provide contact, and fastened to the skin. A ground wire was tightly secured to a screw in the skull. All mice were given a minimum of seven days to recover from surgery before onset of training.

Behavioral Training

Mice were placed into standard laboratory cages different from their home-cage in a sound- and light-attenuated chamber. All WTEB training took place between 0900 and 1400. The headgear described in the surgery section was connected to a tether that was connected to a computer running a custom LabView program. The program delivered both whisker and shock stimuli as well as monitored eyelid closure via a camera attached to the tether. One day prior to training, mice were habituated to the tether and chamber for 10 minutes. On training days, mice were conditioned as previously described (Galvez et al., 2009). A presentation of the CS (250ms whisker stimulation) was paired with a US (100ms periorbital shock, 0.1-0.5 mA square wave shock, 60Hz, 0.5ms pulses). The US shock intensity was tailored to each mouse to generate a detectable eye-blink response with minimal voltage. The CS and US were separated by a 250ms stimulus-free trace interval (Figure 11). Mice were presented with the CS-US pairings 30 times per session (day) with an intertrial interval of 15-25 seconds (mean of 20s). To monitor eyelid closure a camera on the tether provided a live video feed of the eye that was converted to a binary image in LabView in real time. Upon closure of the eyelid, the size of the visible eye

decreased indicating a blink (Figure 11). A conditioned response (CR) was defined as a 4-standard deviation change in size of the eye binary image from baseline, occurring after CS onset and within 20ms prior to US onset (Figure 11). Baseline was defined as the average size of the eye binary image 60ms prior to CS onset for each trial. These settings are consistent with that used in other laboratories conducting eyeblink analyses (Moyer et al., 1990, Tseng et al., 2004, Weiss and Disterhoft, 2011). Mice received one training session per day until behavioral criterion was achieved. Behavioral criterion was defined as exhibiting three CRs out of five consecutive trials for the acquisition group (ACQ). For the criterion group (CRIT), behavioral criterion was defined as exhibiting four CRs in five consecutive trials. For the overtrained (OT) group, behavioral criterion was defined as exhibiting four CRs out of five consecutive trials for two consecutive days. Previous studies from our laboratory have demonstrated these behavioral criterion points to be consistent with acquisition of the trace association (Chau et al., 2014b, Loh and Galvez, 2014, 2015). For behavioral analysis, mice were standardized to the day they reached criterion (C), the two days immediately preceding (C2 = two days before criterion, C1 = one day before criterion), and for the overtraining group, the day following (C + 1 = one day following criterion) to generate learning curves that could be directly compared. Pseudo-trained mice were provided 30 unpaired presentations of the CS and US per day and yoked to an individual trained mouse to control for stimulation induced plasticity unrelated to learning. A cage control group (CC) was also used to establish differences due to surgical procedures. Upon achieving the behavioral criteria established for their group, mice were allowed to complete the training session and then sacrificed after one hour.

Immunofluorescence

For examining learning-induced changes in PD and SOM expression, 7 groups were utilized (CC, Pseudo-ACQ, Pseudo-CRIT, Pseudo-OT, ACQ, CRIT, and OT). One hour after reaching their group specific behavioral criteria, mice were transcardially perfused with 0.1M PBS and 4% paraformaldehyde. The brains were then collected and placed into 4% paraformaldehyde overnight at 4º C and transferred to 30% sucrose in 0.1M PBS until sectioning. Brains were then coronally sectioned at 30µm and stored in cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1M PBS).

Proteins were visualized in the brain sections using a standard immunofluorescence protocol. Free floating sections (3/mouse, spanning S1) were washed in PBS, placed in PBS+ (3% Natural goat serum and 0.5% Triton-x in PBS) for 30 minutes at room temperature and then incubated for 2 days at 4º C in a primary antibody cocktail containing (anti-prodynorphin 1:250, made in rabbit, and anti-somatostatin 1:50 made in guinea pig, AbCam, Cambridge, MA). The sections were then washed in PBS+ and incubated for 2 hours in a secondary antibody cocktail (Goat-Anti-Rabbit AlexaFluor 488 1:100, Goat-Anti-Guinea Pig AlexaFluor 633 1:100; Fisher Scientific) at room temperature. The tissue was then placed into a DAPI solution (300nM; Fisher Scientific) at room temperature for 5 minutes before being dipped in PBS followed by ddH20 and mounted on slides with Prolong Diamond (Fisher Scientific)

Imaging

Slides were imaged using LSM700 (Zeiss Confocal, 40x magnification) at the University of Illinois Institute for Genomic Biology. S1 was localized within each section using the DAPI cellular staining and atlas images (Franklin, 2008). The tissue was then excited with lasers corresponding to the excitation frequency of the secondary antibodies (488nm, 633nm).

Acquired images were analyzed for coexpression with ImageJ by observers blind to the groups. DAPI+ cells provided the locations for determining presence of SOM or PD and each cell was counted as SOM+/SOM- and PD+/PD-.

Statistics

A one-way analysis of variance (ANOVA) will be used to analyze coexpression profiles between groups. In addition, a two-way ANOVA with repeated measures and degrees of freedom adjusted via Kenward-Rogers will be used to analyze behavioral performance across successive days with Bonferroni correction for multiple comparisons.

Results

Behavioral

Mice trained on WTEB conditioning were found to have a significant overall effect of group (Trace or Pseudo; ACQ, CRIT, OT) [F (5, 42.7) = 22.59, $p < 0.0001$], criterion day (C2, C1, C, C+1) [F (3, 70.4) = 23.38, $p < 0.0001$], and a group by criterion day interaction [F (9, 63.6) = 8.33, $p < 0.0001$]. Post-hoc analyses to determine individual differences demonstrated a significant effect between trace and their yoked pseudo groups at ACQ, CRIT and OT behavioral time points. These findings demonstrate that unlike the pseudo group, the trace groups exhibited a sequential increase in acquisition for the trace association (Figure 17).

Somatostatin

For the immunofluorescence analysis, there were a total of two antibodies used (PD and SOM). DAPI was used to localize individual cells, providing sites for analysis of the other two cellular markers, discussed below. There was a significant difference in the number of DAPI+ cells per group, with post-hoc analysis revealing the only difference to be between CC and Trace-Criterion groups. To control for slight differences in cell density, subsequent analyses of

the number of somatostatin positive (SOM+) or prodynorphin positive (PD+) cells were expressed as a percentage of the total number of DAPI+ cells. Using this analysis it was further determined that there were no significant differences in the percentage of DAPI+ cells that were SOM+ between any groups, with no significant effect of neocortical region (top, middle, or bottom), or interaction between SOM+/DAPI+ count and region (Figure 18).

Prodynorphin

Analysis of prodynorphin demonstrated a significant overall effect of the percent of PD+ per DAPI+ cells between groups [F (6, 18.9) = 85.80, $p < 0.0001$], with post-hoc effects demonstrating a significant increase in the percentage of PD+/DAPI+ cells in the trace acquisition (32.10 ± 1.01), and criterion (30.77 ± 1.08) groups relative to pseudo acquisition (13.43 ± 1.05), pseudo criterion (11.29 ± 1.06), and cage controls (9.01 ± 1.07) (Figure 18). However, there was no significant effect of neocortical region (top, middle, or bottom), or interaction between group and region.

Prodynorphin and Somatostatin

Our subsequent analysis of the number of SOM+ cells that were also PD+ demonstrated, consistent with previous reports (Sohn et al., 2014), that 90.85% of PD+ cells were SOM+ (Figure 18). Based on this finding, we further examined the percentage of SOM+ cells that were PD+ or PD- in our training groups and found a significant effect of group on the percentage of PD+/SOM+ cells [F (6, 19.8) = 35.97, $p < 0.0001$] as well as an opposite effect in PD-/SOM+ cells [F (6,19.3) = 24.94, $p < 0.0001$] (Figure 18). There was again no significant effect of neocortical region, or group by neocortical region interaction. Post-hoc analyses further demonstrated that the trace acquisition (57.52 ± 2.79) and the trace criterion (54.82 ± 2.87) groups displayed a significantly higher percentage of PD+/SOM+ cell count than pseudo

acquisition (24.04 ± 2.84), pseudo criterion (21.38 ± 2.86), and cage control (16.43 ± 2.86) groups (Figure 17). The percentage of PD-/SOM+ showed an inverse effect, with the trace acquisition (39.74 ± 3.31) and trace criterion (41.57 ± 3.40) groups displaying a significantly lower percentage than pseudo acquisition (71.34 ± 3.36), pseudo criterion (74.23 ± 3.38), and cage control (82.05 ± 3.38) groups (Figure 18). There were no significant differences noted in the OT groups relative to their appropriate controls in either of these measures. These findings suggest that this increase in PD in SOM neurons is a transient increase that returns to control levels with overtraining.

Discussion

To explore the role of KOR on associative learning, the current study set out to examine learning-induced upstream mechanisms mediating KOR activation. In these studies, it was found that learning induces a significant transient increase in the percentage of neocortical SOM+ cells expressing the precursor peptide PD. Specifically, the current studies found that the percentage of neocortical PD+/SOM+ cells significantly increased during acquisition and into consolidation of the associative paradigm WTEB conditioning. Consistent with these findings, it was subsequently demonstrated that the percentage of neocortical PD-/SOM+ cells decreased during these learning phases. These findings, along with our additional analyses demonstrating that the percentage of SOM+ cells remained constant with learning further suggests that learning does not alter neocortical SOM expression but rather PD expression within SOM+ cells. Interestingly, our subsequent analyses of the percentage of neocortical PD+/SOM+ cells during overtraining demonstrated a significant reduction to pre-conditioning expression levels. These findings further support a learning-induced time-dependent neocortical regulation of neocortical PD expression in SOM+ neurons.

Our previous findings have shown that neocortical KOR plays a prominent role in facilitating acquisition and consolidation of the forebrain dependent paradigm WTEB. Specifically, we have shown that KOR antagonism can dramatically impair acquisition (Loh and Galvez, 2015) while selective antagonism of the different phases of KOR activation (driving kinase or transcription factor activation) can independently impair either acquisition or consolidation of the association (Loh et al., 2017). The current study provides the first examination of the endogenous mechanisms driving this learning-induced KOR activation. KOR's endogenous ligand, dynorphin, is derived from the precursor PD. Interestingly, studies have shown that PD's precursor, PPD in somatosensory cortex is predominately expressed in SOM+ cells (Sohn et al., 2014). Our findings are consistent with these findings as 90.85% of PD containing cells were also found to express SOM. Interestingly our subsequent findings demonstrating that KOR activation plays a transient role in neocortical learning is also consistent with our previous study demonstrating that different phases of KOR activation facilitate different phases of learning (Loh et al., 2017). Furthermore, these findings are consistent with previous studies from our lab (Chau et al., 2014b) demonstrating that learning results in a neocortical synaptic reorganization that returns to pre-conditioning levels with overtraining. Collectively these studies provide a comprehensive time-dependent, cell-specific understanding of a neocortical mechanism mediating associative learning.

We should note, although our findings that PD+/SOM+ expression increases during learning is novel and greatly adds to our understanding of KOR in associative learning, one must be cautions regarding the global implications of these findings. The current study focused on PD expression during a specific associative learning paradigm in a single neocortical brain region. PD expression in other brain regions during different learning paradigms has not been

explored and should be a focus for subsequent studies. Furthermore, while we were able to detect a transient increase in the percentage of PD+/SOM+ cells with learning, it would be of interest for future studies to utilize a higher temporal resolution in exploring the time- and learning-dependent neocortical mechanism mediating task acquisition and memory consolidation.

Interestingly, contrary to our findings others have found that learning can alter neocortical SOM expression. Cybulska-Klosowicz et al., found that the number of SOM+ cells was increased within layer IV neocortical whisker barrels 24 hours following a three-day conditioning procedure (Cybulska-Klosowicz et al., 2013). Unlike the current study, this study utilized a delay learning paradigm, where whisker stimulation was paired and co-terminated with a tail rather than periorbital shock US. Further studies would be needed to determine if these differences were the cause for the increased SOM expression and if this could then alter KOR activation. Interestingly in addition this discrepancy with learning induced SOM expression, others have found that PD knockout mice exhibit heightened freezing following fear conditioning (Bilkei-Gorzo et al., 2012). Furthermore, hippocampal CA3 dynorphin injections impair water maze conditioning escape latency, but not retention performance (Sandin et al., 1998). Although these studies appear to contradict the current findings, the analyses by Bilkei-Gorzo et al (2012) were conducted with genetic knockout mice that could have abnormal neuronal development due to the absence of the gene from utero. Furthermore, Sandin et al (1998) explored the role of dynorphin in the CA3 region of the hippocampus, while the current study explored dynorphin's precursor in S1, making it difficult to draw a direct comparison between the two studies. Future studies would be needed to better understand these possible differences and more fully elucidate the process by which this mechanism is capable of

modulating acquisition and memory consolidation for different learning paradigms in different brain regions.

The current study identified a possible upstream driver of neocortical KOR regulation with associative learning in S1. Interestingly, the transient increase in PD+/SOM+ cells suggests that there is an increase in PD synthesis in cells that were otherwise relatively dormant prior to the learning event. Furthermore, the fact that this increased expression returns to baseline with overtraining further solidifies the time dependency of this process. In the growing field of KOR mediated learning, the current study identifies an important upstream component that can be used to better understand the mechanisms by which we form associations.

Figures

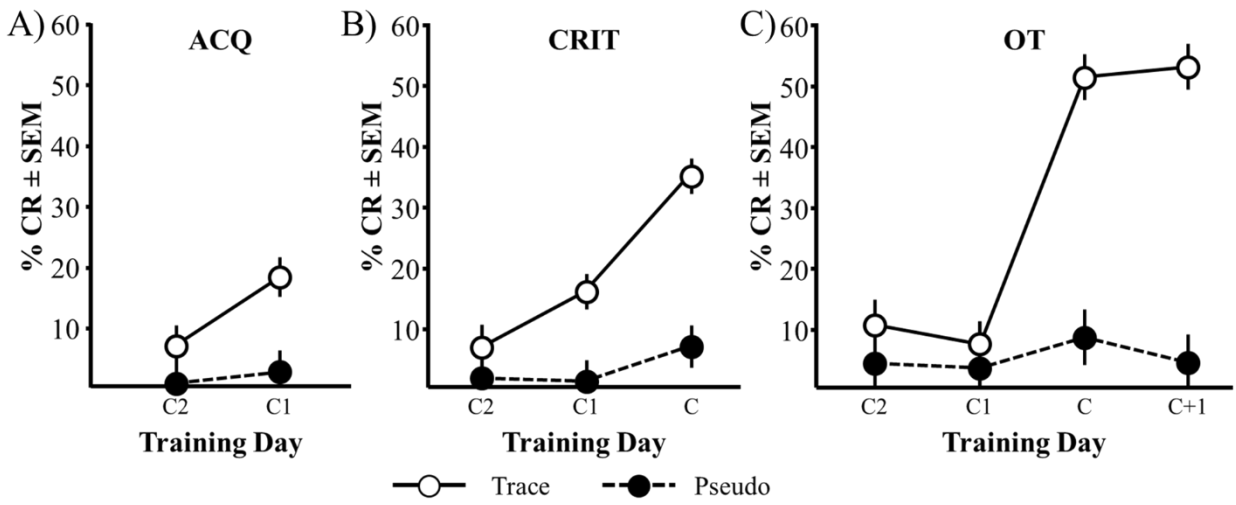

Figure 17: Behavioral graphs representing the percent conditioned responses (%CR) across training days (C2 = 2 days before criterion, C1 = 1 day before criterion, C = day of criterion, C+1 = 1 day after criterion). All trace groups performed significantly better than respective pseudo groups. All trace groups significantly acquired the association over time, with increasing %CR with successive days and pseudo groups not exhibiting significant differences across training days. ACQ = Acquisition, CRIT = Criterion, OT = Overtrained.

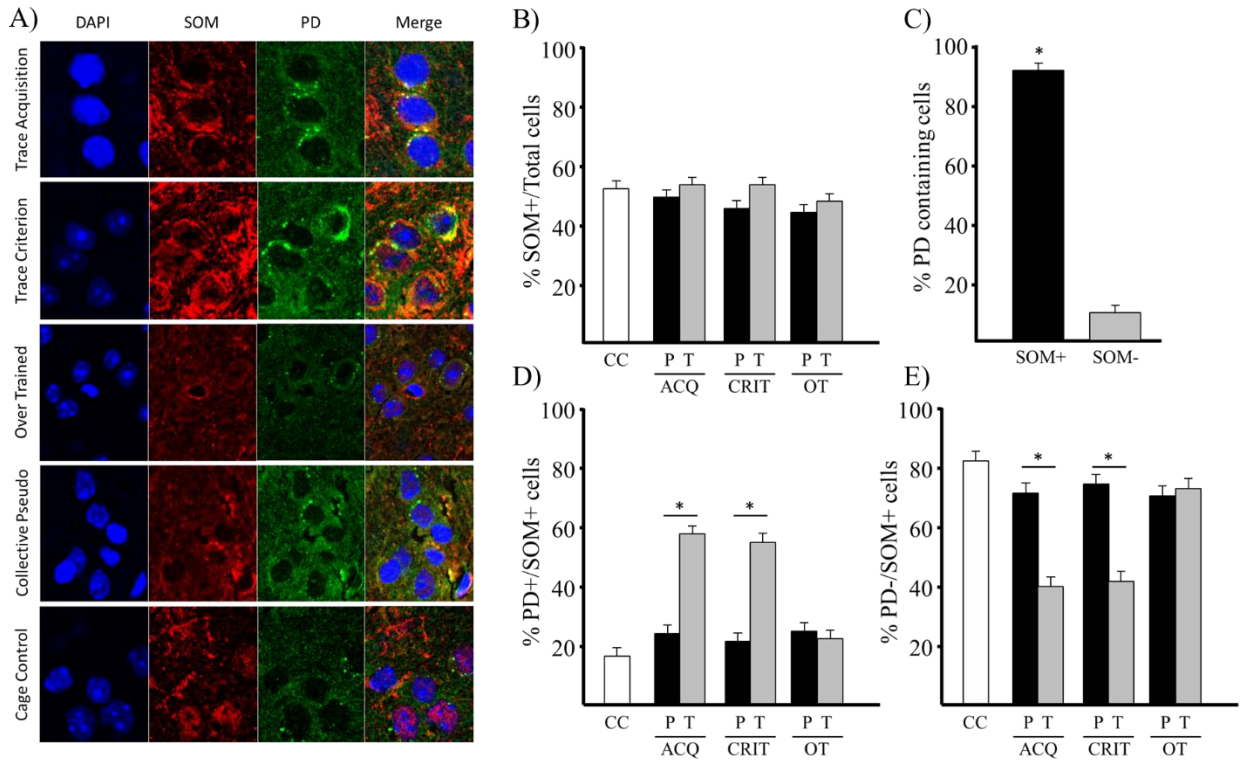

Figure 18: Immunofluorescence expression across learning in various groups. A) Representative fluorescent images for individual groups and channels. B) Percentage of total DAPI containing cells that expressed Somatostatin (SOM). There was no significant difference between Pseudo (P) and Trace (T) groups in the percentage of cells expressing SOM. C) Percentage of Prodynorphin (PD) cells that expressed SOM. Approximately 90% of PD expression was found in SOM containing cells. D) Percentage of PD+/SOM+ expressing cells of the total DAPI stained cells. The percentage of SOM cells expressing PD significantly increased during the Acquisition (ACQ) and Criterion (CRIT) time points but returned to control levels in the overtrained (OT) group. E) Percentage of SOM cells that did not express PD. There was a significant decrease in the percentage of SOM cells not expressing PD during ACQ and CRIT, but similar to the findings in D, returned to control levels with OT.

Chapter 6 – Overall Conclusions

The studies described in this dissertation collectively examine the role for KOR and its up- and downstream components in a neocortical-dependent learning task. In chapter 2, we demonstrated that following pre-training injections of the general opioid antagonist Naloxone, mice are significantly impaired in their ability to acquire the WTEB association. The subsequent study outlined in chapter 3 demonstrated that following either systemic or local S1 administration of the KOR specific antagonist NorBNI, mice displayed significantly impaired acquisition for the WTEB association. Focusing on better understand the relationship between neocortical KOR activation and learning, chapter 4 demonstrated that blocking the initial phase of KOR activation in S1, that drives activation of various kinases such as PI3K, PKC, and ERK1/2, significantly impaired acquisition for the WTEB association. Furthermore, in chapter 4, it was demonstrated that blocking the later phase of KOR activation, that drives activation of the transcription factors CREB and zif268, selectively impaired consolidation of the WTEB association. To obtain a better understanding of this learning-induced process, the studies outlined in chapter 5 demonstrated a possible upstream regulator of learning-induced neocortical KOR activation, PD expression in SOM neurons. These studies demonstrated that the proportion of PD+/SOM+ cells transiently increased in S1 during the acquisition and criterion phases of WTEB. Interestingly, this increase in PD expressing cells in S1 was limited to a short time-period around the learning event, as mice that were trained beyond the criterion time point (overtrained) did not display this increase.

The findings in this dissertation, along with our prior analyses and those from other laboratories collectively provide a comprehensive previously unexplored role for KOR in a neocortical mechanism mediating learning. These studies suggest that during the initial phases

of learning, neocortical GABAergic activation is increased. Specifically, it was found that learning increases neocortical GAT-1 expression (a GABAergic marker) (Siucinska et al., 2014). Interestingly these findings are consistent with our studies demonstrating that learning increases the percentage of PD+ expressing inhibitory neurons; thus, further suggesting that this increased GABAergic activation is increasing PD expression. Increases in PD, are likely to cause increases in dynorphin expression, as seen in other areas of the cortex (Romualdi et al., 1999); thus, further suggesting increased KOR activation.

KOR activation will initially result in phosphorylation/activation of various kinases shown to have a direct role in learning and task acquisition. For instance, antagonizing PI3K in CA3 of the hippocampus has been shown to block acquisition for conditioned place preference (Cui et al., 2010). Similarly, antagonizing PKC within the mPFC disrupts novel object recognition in short (10 min), but not long (24 hour) delays (Evuarherhe et al., 2014). Additionally, ERK1/2 has been shown to be upregulated in the anterior cerebellar vermis as early as two minutes following the third session of delay eyeblink conditioning in rabbits (Zhen et al., 2001). Consistent with these studies, it was demonstrated in Chapter 4 that blocking this initial kinase activation in S1 significantly impairs acquisition for the WTEB association but did not impair long term memory consolidation (Loh et al., 2017). Interestingly our laboratory has also shown that this initial phase of learning is associated with an increase in neocortical dendritic spines (Chau et al., 2014b). Thus further suggesting a possible link between KOR-dependent kinase activation and dendritic spine proliferation.

Following initial KOR activation and subsequent stimulation, KOR activates the transcription factors pCREB and zif268. CREB has been shown to be necessary for long term memory consolidation. Specifically, CREB conditional knockouts displayed significantly

reduced freezing 24 hours following fear conditioning, but not 2 hours following (Kida et al., 2002). Additionally, CREB antisense administration before water maze training caused increased escape latency 48 hours after acquisition, while not having any effect on initial acquisition (Guzowski and McGaugh, 1997). Similarly, zif268 has been demonstrated to increase expression in the mPFC from 1 to 30 days following water maze training (Barry et al., 2016). Consistent with these findings, the studies in Chapter 4 demonstrated that blocking this late-phase KOR-dependent transcription factor activation inhibits WTEB consolidation. Prior learning-induced anatomical studies from our laboratory have further demonstrated a conditioning induced increase in dendritic spine density with WTEB during the acquisition and consolidation time points, thus further suggesting synaptic reorganization (Chau et al., 2014b). Collectively these findings suggest that this KOR-dependent transcription factor activation is facilitating synaptic plasticity, consolidating the learned information for long term storage.

Interestingly our findings further demonstrated that following behavioral criterion (overtraining) the percentage of PD+/SOM+ cells decreased back to baseline levels. This decrease in PD expression would likely cause a decrease in DYN concentration, as seen in the hippocampus (Thai et al., 1992), thus reducing KOR activation. Likewise, we found that neocortical KOR expression is significantly reduced during behavioral criterion, immediately prior to the reduced PD expression, suggesting receptor internalization and downregulation (Loh et al., 2017). Studies from our laboratory have further demonstrated that learning induced increased dendritic spine density on spiny stellate cells in S1 returns to preconditioning levels during this overtraining period (Chau et al., 2014b). Given the proposed importance for this mechanism in learning and memory consolidation, down regulation and removal of the receptor at specific synapses mediating the new memory is an interesting mechanism that would greatly

facilitate acquisition and consolidation of a single memory, while minimizing distractions from other new memories. Further studies will be needed to determine the potential role for this receptor downregulation with learning along with the time course for its return to pre-training expression levels. For an overall summary of the process outlined in the above paragraphs, see Figure 19.

While this dissertation outlines one of many possible neocortical learning mechanisms, KOR represents an under explored, yet unique mechanism in its ability to unilaterally activate/regulate several already known and accepted learning mechanisms. The outlined mechanism has been characterized within S1, however other brain regions such as the motor, auditory, and orbitofrontal cortices display similar expression profiles of PPD+/SOM+ cells (Sohn et al., 2014), suggesting a possible similar learning-dependent KOR mechanism in these regions. However, the current dissertation is, of course, not without limitations. WTEB induces neuronal plasticity in S1; yet, these changes do not exist in a vacuum as there are many brain regions capable of influencing S1. For instance, the amygdala can alter stimuli salience entering S1 and has been shown to be capable of facilitating initial acquisition for delay eyeblink conditioning, and to a lesser extent, trace eyeblink conditioning (reviewed in Chau et al., 2012). Additionally, while the current dissertation offers a detailed explanation of one mechanism of learning in one brain region, in order to better generalize this mechanism to other paradigms/brain regions, future work would be required that utilizes the plethora of available behavioral paradigms and animal models.

The current dissertation outlines a specific neocortical KOR dependent mechanism mediating trace-associative learning. The findings described in this dissertation are novel and offer a significant advancement in our understanding of how memories are formed and stored

within the neocortex. It is the view of the author that the mechanism outlined in this dissertation provides the most compelling evidence for a role of KOR in associative learning.

Figure

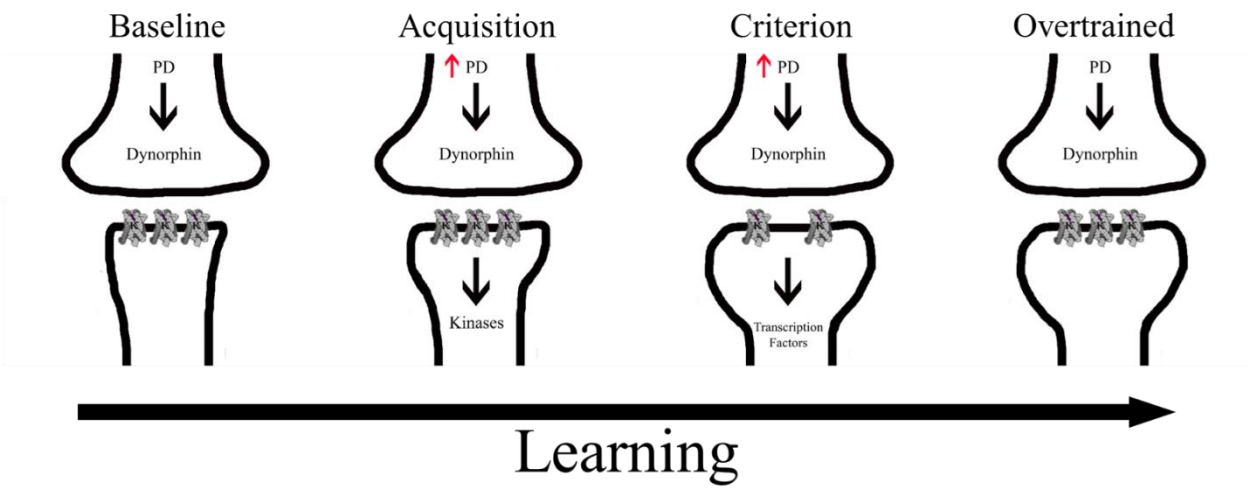

Figure 19: General summary of the proposed mechanism for KOR in S1 with learning. During baseline prodynorphin (PD) is used to synthesize dynorphin which will be released and activate the Kappa Opioid Receptor (KOR) at specific synapses. During exposure to whisker trace eyeblink conditioning (Acquisition), there is an increase in the level of PD, which will likely increase downstream activation of KOR, and facilitate initial restructuring of dendritic spines on spiny stellate cells. Once mice reach behavioral criterion, the increased levels of PD are sustained, and further restructuring of dendritic spines occurs. Furthermore, during this time point there is a significant downregulation of KORs, likely associated with ligand-induced internalization and degradation. Additionally, during this time period there is an increase in KOR mediated transcription factor activation (phase 2 of KOR activation). Once mice have reached overtraining (2 consecutive criterion days) the PD expression returns to baseline, and the KOR expression likely returns to baseline. An important note about the figure; while we have direct evidence of spine changes as a result of learning, and direct evidence for KOR and its up/down stream components changing, we do not have direct evidence of these two mechanisms occurring at the same location; however, for simplification purposes in the summary, the two mechanisms

Figure 19 (cont): were combined. Future work will be required to determine if these mechanisms are working in concert, or completely separately to form new associations.

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